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A Study of the Carbohydrate Specificity of Hyperimmune Fowl Globulins

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A STUDY OF THE CARBOHYDRATE SPECIFICITY OF HYPERIMMUNE FOWL GLOBULINS

A thesis submitted by

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TABLE OF CONTENTS

SUMMARY

Past studies of the immunochemistry of avian species have often presented conflicting results. Confusion has arisen from failure to account for the role of macroglobulin antibody and from the aggregation of gamma globulins in 8% NaCl solutions. It has been found that the production of macroglobulin antibodies in chickens diminishes with repeated antigenic stimulations. Accordingly, this study was made of the carbohydrate specificity of hyperimmune fowl globulins where immunological reactions were carried out in physiological saline. This was the first study of the carbohydrate specificity of fowl immune globulins and was the first well-defined avian system where macroglobulin antibodies were eliminated or significantly reduced and the aggregation of gamma globulins in high salinity avoided.

Glucose, xylose, and cellobiose groups were coupled to bovine serum albumin by a phenylazo linkage to form synthetic antigens. This was the first known use of xylose as an antigenic determinant group. Chickens were injected with solutions of each of these antigens and blood samples were collected after repeated immunizations. Plasma samples containing antibodies were pooled and studied in immunological reactions.

Fractionation of pooled plasma samples resolved macroglobulin components, gamma globulins, and albumin. Antibody activity was associated only with 7.5S gamma globulins. It was concluded that hyperimmune chicken plasma contained no precipitating macroglobulin antibodies.

Precipitin tests with plasma obtained in response to each of the synthetic antigens exhibited specificity for the homologous carbohydrate group. The response of precipitating anticarbohydrate antibody while specific was very low. The response against β-D-glucoside and β-D-cellobioside determinants was much lower

than the response in rabbits to these same groups. Thus, chickens were quantitatively inferior producers of precipitating antihapten antibodies.

Quantitative hapten inhibition studies with antibodies specific for the p-azophenyl-β-D-cellobioside residue demonstrated the relative importance of each portion of the antigenic determinant in conferring specificity. These were in decreasing order of importance:

the terminal nonreducing glucose group

the β-linkage between glucose units

the cellobiose group

the position of the β-linkage between glucose units

the p-diazophenyl group

It was concluded that the specificity characteristics of chicken antibodies were similar to those of rabbits.

INTRODUCTION

FOWL IMMUNOCHEMISTRY

When a vertebrate animal is injected with a suitable foreign material there appears in the bloodstream within a few days substances possessing the unique property of reacting with the material injected. These substances are antibodies and the foreign material that stimulated their production are antigens. Immunochemistry is the study of the chemical nature of antigens and antibodies and the chemical basis of their reactions.

The immunochemistry of avian species only recently has begun to be clarified. The literature indicated some of the ambiguities which existed when the present work was undertaken.

Wolfe and his associates at the University of Wisconsin have studied the immune response in chickens since 1942. From their first publication it was apparent that chicken antiserum, the fluid portion of the blood containing antibodies, did not behave normally. Using chicken antisera against whole sheep, bovine, or buffalo serum, Wolfe (1) found that the antisera strength as measured by interfacial titer increased markedly when aged for nine or more days. Rabbit antisera to the same whole sera did not show a titer change on aging.

Subsequently, Goodman, et al. (2) found that the amount of precipitation of chicken antisera with serum protein antigens was highly dependent on the concentration of NaCl. Although serological reactions are normally carried out in physiological saline (0.15M NaCl, ca. 0.9%), they suggested that salt concentrations of 8% were necessary for a more complete precipitation of specific antibody. The increased precipitation at 8% NaCl was not the result of salting out of serum proteins which were known to precipitate in 14-18% NaCl. With mammalian antibodies,

increasing the salt concentration above 1% in precipitin tests results in a decrease in the amount of precipitated antibody.

The aging phenomenon was again studied by Gengozian and Wolfe (3). Primary antiserum, serum obtained in response to a single intravenous injection of antigen, was aged at 4°C. and -20°C. for one week. Precipitate formation was observed in aged or frozen and thawed antisera. Aged sera showed higher interfacial titers, as observed previously, but decreased titers in quantitative precipitation studies when compared with fresh sera, demonstrating that the two test methods were not comparable. They also found a coprecipitation of nonspecific material with specific antigen-antibody complexes. They concluded that this nonspecific material was not complement (a group of thermolabile components found in serum which is specifically required for certain antigen-antibody reactions). Decomplementation of the serum with disodium ethylenediaminetetraacetate (EDTA) had no effect on precipitin tests made in 8% NaCl. In 1% NaCl, however, EDTA treatment or heat inactivation of fresh or aged antisera resulted in reduced precipitation as compared with untreated control sera. There was apparently coprecipitation of complement in 1% saline.

Free boundary electrophoresis was used by Deutsch, et al. (4) to study the antibody response of chickens to repeated injections with human γ_2 globulins. They thought that both alpha and gamma chicken globulins were precipitated by the human γ_2 globulins. Normally, antibodies are considered to have only gamma electrophoretic mobility. Ethanol fractionation of their sera with subsequent precipitin tests showed only traces of antibody activity in the albumin and alpha globulin fraction and a reduced activity in the gamma globulin fraction. Recombination of their ethanol fractions produced more precipitation than the original unfractionated serum. These workers concluded that the alpha globulins coprecipitated with the specific antigen-gamma globulin antibody complexes.

Banovitz and Wolfe (5), in a similar study, found no evidence for coprecipitation of alpha globulins and criticized previous evidence based on free boundary electrophoresis. Also, the ethanol fractionation procedure used by Deutsch, et al. (4) resulted in turbid, unstable fractions, which probably explains why their reconstituted serum produced more precipitation than their original unfractionated serum in quantitative precipitin tests.

Coprecipitation of a normal serum protein with antigen-antibody complexes was observed by Makinodan, et al. (6). They studied primary and secondary serum obtained in response to injections with bovine serum albumin (BSA). A serum macroglobulin with 21S sedimentation coefficient and beta or gamma electrophoretic mobility and salting-out properties coprecipitated with BSA-antiBSA aggregates in 1.5M NaCl regardless of the age of the chicken antiserum. In 0.15M NaCl coprecipitation occurred with aged antiserum but not with fresh. They also noted that associated with aging was the formation of a gelatinous or flocculated material.

Orlans and coworkers (7-12) in a series of papers made an extensive study of fowl complement and antibody precipitation reactions. They too worked with primary and secondary antiBSA sera and observed coprecipitation of a macroglobulin component. They could not demonstrate that the macroglobulin substance was a component of complement. They did conclude that the macroglobulin was not a high molecular weight antibody and was not responsible for the increased precipitation of fowl antisera at high salt concentrations. There were more macroglobulins present in specific precipitates made in 0.9% NaCl than in 8% NaCl.

The effect of primary, secondary, and tertiary injections of BSA on chicken antiserum production was studied by Benedict, et al. (13). They followed antibody production by the passive hemagglutination (HA) technique. The method is a qualitative measure of the ability of the serum to agglutinate or clump red blood cells

on which the immunizing antigen has been adsorbed. The titer is defined as the reciprocal of the highest dilution of the antiserum where agglutination is still detected. The antisera were fractionated on a diethylaminoethyl (DEAE) cellulose column and the fractions examined in the analytical ultracentrifuge and for HA activity.

They found that peak HA titers were obtained 6 days after the primary injection and thereafter titers dropped rapidly. Secondary and tertiary stimulations increased peak titers but not to the same extent found with the primary stimulation. The ultracentrifuge resolved both high molecular weight, 19S, and low molecular weight, 7S, antibodies in the DEAE column fractions. In primary antisera, HA activity was associated only with the 19S macroglobulins. Analysis in the ultracentrifuge of antigen-antibody complexes formed in 8% NaCl using primary antisera indicated that about 60% of the protein precipitated was 19S globulin.

They also obtained a rough estimate of the ratio of high to low molecular weight HA antibody in their antisera during the course of injections. Each immunization produced an initial increase in the ratio of high to low molecular weight antibody. Following each injection this ratio decreased over a ten-day period, then remained at a nearly constant value. The peak ratio in primary antisera was about 200:1 while in tertiary antisera it was only about 1:1. Apparently, the rate of production of macroglobulin decreases with each subsequent injection while the synthesis of 7S globulins increases.

Říha (14) found that chicken macroglobulin antibodies were much more active in hemagglutination reactions than 7S globulins and were far less specific. The macroglobulins in primary, secondary, and hyperimmune sera (the latter obtained after repeated injections with antigen) all showed cross reactivity and little inhibition by homologous antigen in HA reactions. That is, macroglobulins formed in response

to BSA would cross react with human serum albumin and were not greatly inhibited in HA reactions by the addition of BSA. Macroglobulin antibodies were extremely sensitive when isolated and tended to precipitate spontaneously. Ríha could not demonstrate specific precipitating antibody of the macroglobulin type.

However, the 7S globulins were very specific in HA tests; little cross reactivity and considerable inhibition by homologous antigen. The highest amount of precipitating 7S antibody was found in hyperimmune serum. Hyperimmune antibodies also had the greatest specificity in HA reactions.

The reason for increased precipitation of chicken antiserum in 8% NaCl was explained in a paper by Hersh and Benedict (15). They found that chicken 7S globulins in 1.5M NaCl formed either a tri- or tetramer. Rabbit gamma globulins in 8% NaCl or chicken gamma globulins in 0.15M NaCl did not aggregate. Aggregation of normal chicken globulins with immune gamma globulins at high salinities produces the increased precipitation with antigen, as originally observed by Goodman, Wolfe, and Norton (2).

CARBOHYDRATE SPECIFICITY OF ANTIBODIES

Immunochemistry is concerned with the chemical configuration of a molecule or a portion of a molecule that elicits an antibody response against it, the antigenic determinant. It is also concerned with the polypeptide chains of a gamma globulin molecule that are folded into a region complementary in configuration to the antigenic determinant, the antibody combining site. The degree of complementarity or the closeness of fit between the antigenic determinant and the antibody combining site determines the specificity of the antibody.

The basis of modern immunochemistry resides in the pioneering efforts of Heidelberger and his coworkers (16) and Landsteiner (17). Heidelberger, et al.

developed the quantitative methods for assaying antigens, antibodies, and complement. Landsteiner introduced low molecular weight antigenic determinants of known chemical structure into antigenic proteins and developed the hapten inhibition technique. A hapten is a substance which on injection does not produce antibodies but can react with antibodies specifically to produce or to inhibit precipitation. Hapten inhibition is a measure of the ability of a low molecular weight hapten to compete for the antibody combining site and inhibit the precipitation reaction with antigen.

There have been innumerable immunochemical studies made on carbohydrate specificity. The reviews of How, et al. (18) on the pneumococcal polysaccharides and of Watkins (19) on blood group substances are cited as examples of two important areas where carbohydrate specificity has been extensively studied. The review here is concerned only with studies where mono- and disaccharides coupled to a carrier protein form an antigenic determinant. Hapten inhibition studies made with antibodies to these antigenic determinants have provided information on the size and chemical requirements for specificity of the antibody combining site.

Early studies by Goebel, et al. (20-23) established that the immunochemical specificity of carbohydrates was determined by their chemical configuration. In all of their studies the carbohydrate substances were coupled to a protein substrate by an azophenyl linkage. Rabbits were immunized with these antigens and antisera were tested with homologous and heterologous carbohydrates coupled to protein substrates other than those used for immunization. A comparison of a β-glucoside with β-galactoside (20) demonstrated that an interchange of H and OH on the fourth carbon atom in the pyranose ring conferred immunological specificity. Cross reactivity was observed when the change was on the first carbon atom; α-glucoside compared with β-glucoside, though the antibodies were predominantly specific (21). Introduction of an acetyl group at the sixth carbon atom again conferred a distinct specificity

(22). Comparison of antisera to maltose, cellobiose, lactose, and gentiobiose indicated that immunological specificity was determined by the terminal non-reducing sugar molecule, the position of linkage between the sugar units, and the glycosidic molecule as a whole (23). The configuration of the terminal hexose molecule was most important in determining serological cross reactions. All of the work by these authors was based on qualitative observations of precipitation in antisera with varying dilutions of test antigen. While their conclusions were probably correct they were not based on a firm quantitative foundation.

Kabat and his coworkers (24-27) studied human and rabbit antisera to dextrans, high molecular weight polymers of D-glucose with predominantly α-(1,6) linkages. Hapten inhibition studies with the series of α-(1,6) linked oligomers of D-glucopyranose showed that in most antidextran systems isomaltohexaose and isomaltoheptaose gave maximum and nearly equivalent inhibition on a molar basis (24, 25). The antidextran combining sites apparently are complementary to terminal chains of up to six or seven α-(1,6) linked glucose units. Further studies on antidextrans with α-(1,6) specificity indicated that the substituents at the second and third carbons in the terminal pyranose ring were more important in the binding to the antibody combining site than the substituents on carbon six (26). Because dextrans are complex, highly branched polymers, the chemical configurations of the antigenic determinant groups were unknown.

Arakatsu, et al. (27) did study the response of rabbits to an α-glucosyl and an α-isomaltosyl determinant. They coupled isomaltonic and isomaltotrionic acid to BSA by the mixed anhydride reaction. Antiserum produced in response to the α-glucosyl determinant showed α-glucosyl specificity but would not precipitate with dextrans. Antibodies to α-isomaltosyl were precipitated by dextrans. In hapten inhibition studies isomaltotriose, isomaltotriitol, and isomaltotrionic acid

gave equivalent molar inhibition of dextran precipitation. The configuration at the first carbon atom on the reducing end of a triose inhibitor apparently has little immunochemical significance in this system. The antibody response was found to be heterogeneous. With most of the antisera, isomaltotriose was the best inhibitor of dextran precipitation. However, with some antisera an isomaltotetraose or isomaltopentaose was required for maximum inhibition. The antibody combining site apparently could accommodate a tetra- or pentasaccharide even though the antigenic determinant was only an α-isomaltosyl residue. This is possible if a portion of the antibody is specific for an internal α-(1,6) glucose sequence. A triose would have only one internal sequence, a tetraose two, a pentaose three, etc. The tetraose and pentaose would have a distinct statistical advantage over the triose in reactions with antibody having such an internal sequence specificity. Another explanation of this phenomenon is that the true antigenic determinant includes the α-isomaltosyl residue, the hexonic acid linkage to an ε-aminolysine, and perhaps also a portion of the carrier protein peptide chain. They concluded that the antibody combining site then would have a large complementary region that could accommodate the larger oligosaccharide.

Yariv, et al. (28) coupled diazophenyl β-glucoside, -galactoside, and -lactoside to phloroglucinol to form polyfunctional precipitating haptens. Rabbits were immunized with the same glycosides conjugated to gamma globulins. The antibodies formed precipitated only with the homologous phloroglucinol glycoside. In hapten inhibition studies the p-nitrophenyl-β-glycosides had equivalent molar inhibition in their respective systems. But the monosaccharides; glucose and galactose, were much weaker inhibitors in their homologous systems than was lactose in the antiazophenyl lactoside system. The authors believed that this was due to the multitude of conformations the free hexose assumed in solution. The bound hexose in the antigenic determinant contains a bulky substituent at the anomeric carbon that could

stabilize one particular ring conformation. The antibody combining site complementary to that conformation would accommodate only that portion of the free hexose inhibitor having a similar conformation.

Finally, Allen and his coworkers (29, 30) have studied antibody response in rabbits to cellobiose and sophorose determinants coupled to BSA by a phenylazo linkage. With both systems the respective disaccharides were better inhibitors on a molar basis than the corresponding tri- or tetrasaccharides. Also, in both systems antibody could readily distinguish between the β-(1,2), -(1,3), -(1,4), and -(1,6) linked glucobioses. Cellobiose, β-(1,4), and sophorose, β-(1,2), were much better inhibitors of antibody precipitation in their respective systems than were any of the other beta or alpha linked glucobioses. In the anticellobiose system oligosaccharides possessing a terminal nonreducing cellobiose unit were more potent inhibitors than compounds with internal cellobiosyl groups. For example, cellobiose and cellotriose were both better inhibitors than the trisaccharide β-D-Glup-(1→3)-β-D-Glup-(1→4)-β-D-Glup. This suggests that there was little antibody present specific for an internal β-(1,4) sequence. In both systems the p-nitrophenyl derivatives were the most potent inhibitors of antihapten precipitation. The azophenyl linkage to BSA was then an important part of the antigenic determinant and conferred specificity on the antibody combining site.

STATEMENT OF THE PROBLEM

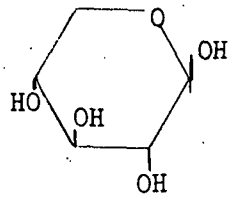
The purpose of this work was to study the carbohydrate specificity of hyperimmune chicken globulins by quantitative precipitin and hapten inhibition tests.

From the literature review it is apparent that many of the difficulties involved with the study of immune fowl globulins arise from the macroglobulins. These globulins predominate in primary or secondary antisera. They appear to be important in interfacial titer and hemagglutination tests and probably also coprecipitate in precipitin tests. The macroglobulins apparently are not highly specific in their combination with antigen. When isolated, the macroglobulins are unstable and readily precipitate from solution. This may account for the formation of gelatinous or flocculated material present in aged chicken serum or in serum that has been frozen and thawed. Since the amount, specificity, and stability of these macroglobulins are nearly always in doubt, in any meaningful study of the chicken immune system they should be eliminated or at least considerably reduced.

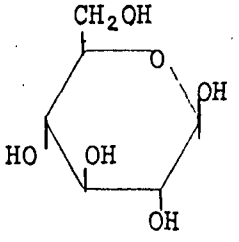
Benedict, et al (13) and Říha (14) have demonstrated that macroglobulin production diminishes with repeated antigenic stimulations. Hyperimmune antibodies should consist primarily of 7S precipitating gamma globulins with only a minimum amount of macroglobulin. Accordingly, the system selected for study was the precipitating antibodies of hyperimmune chicken plasma. All tests are to be made in physiological saline to avoid the gamma globulin aggregation demonstrated by Hersh and Benedict (15).

The antigenic determinants to be studied in this system are β-D-xylopyranosyl, β-D-glucopyranosyl, and β-D-cellobiosyl residues. These groups were selected for their availability and for their relationship to the polysaccharides found in wood

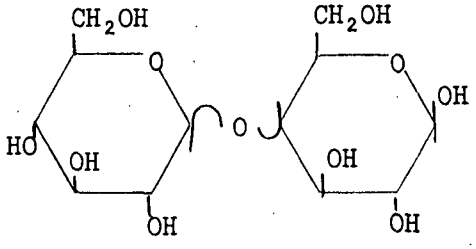
fibers. Also, since glucose and cellobiose determinants had been previously studied in rabbits (20, 21, 23, 27-29), this information was available for comparison with the chicken immune system. The structures to be studied are illustrated below:



β-D-xylopyranose



β-D-glucopyranose



β-D-cellobiose

The β-D-xylopyranose determinant has apparently never been studied before in an immunochemical system. This pentose has the same configuration as β-D-glucopyranose at carbon atoms one to four but does not have the hydroxymethyl group ($-CH_2OH$) at carbon five. A comparison of these two systems should provide information on the immunochemical significance of this hydroxymethyl group.

Hapten inhibition studies in the β-D-cellobiose system should demonstrate the importance of the β anomeric linkage between the glucose units, the point of attachment of the β linkage between the glucose units, and the configuration of the terminal nonreducing glucose unit.

EXPERIMENTAL PROCEDURES

SYNTHESIS OF IMMUNIZING ANTIGENS

Crystalline bovine serum albumin (BSA), 99% pure, was purchased from the Pierce Chemical Company. Disk electrophoresis of BSA by the technique of Davis (31) resolved two distinct protein bands with albumin and prealbumin electrophoretic mobility. There were also trace amounts of alpha globulins present.

p-Nitrophenyl-β-D-glucopyranoside and p-nitrophenyl-β-D-xylopyranoside were also purchased from the Pierce Chemical Company. Both of these compounds were catalytically reduced to the p-aminophenyl derivatives in a hydrogenation apparatus (Parr Instrument Co., Model CA). p-Aminophenyl-β-D-cellobioside was synthesized by the reaction scheme given in Appendix I. All of the p-aminophenyl derivatives were diazotized and coupled to BSA by the procedure of Campbell, et al. (32).

The three azo proteins were lyophilized and samples of each submitted to the analytical department for determination of the carbohydrate content (33). Table I lists the percent of carbohydrate found and the degree of substitution (D.S.) for the three azo proteins.

TABLE I

PROPERTIES OF IMMUNIZING ANTIGENS

Antigen	Carbohydrate, %	D.S.[a]
BSA-p-Diazophenyl-β-D-glucopyranoside	7.6	34
BSA-p-Diazophenyl-β-D-xylopyranoside	3.7	19
BSA-p-Diazophenyl-β-D-cellobioside	5.4	11

[a] Assumed BSA mol. wt. of 70,000; Campbell, et al. (32).

IMMUNIZATION OF CHICKENS

Several groups of white leghorn chickens were used during the course of this study. All chickens were hens at least two years old. Wolfe, et al. (34) studying the response of large groups of chickens to a single injection of BSA could find no statistical difference in antibody response related to chicken sex or breed. The chickens they studied reached serological maturity after 22 weeks while the highest antiserum titers were obtained from chickens 3 to 8 years old.

Each of the azo protein antigens was reconstituted in 0.9% saline solution at a concentration of 10 mg./ml. Groups of chickens were given periodic injections of 1 ml. of azo antigen intravenously (alar vein) and 0.5-ml. injections of Freund's Adjuvant (Colorado Serum Laboratories) subcutaneously. Sterile, disposable 1-cc. tuberculin syringes with a 1/2-inch, 26-gage needle were used for all injections. The first three immunizations were given at one to two week intervals. Thereafter, the chickens received periodic booster injections about once a month.

While several chickens were lost during the course of injections and bleedings, only two deaths were definitely attributed to anaphylactic shock. Other losses probably resulted from ill health prior to immunizations, overcrowding, or "just old age."

PLASMA PREPARATION AND ANTIBODY DETECTION

Blood samples were removed from the alar vein. A 10-cc. hypodermic syringe with a 1-inch, 23-gage sterile needle was used for this purpose. No more than 10 cc. of blood were removed from a chicken at any one bleeding.

A 2% EDTA in saline solution was added immediately to the collected blood samples in a ratio of one part of EDTA solution to ten parts blood. The blood

of mature chickens clots without contracting (35), which traps the plasma, containing the antibodies, within the clot. EDTA chelates the calcium ions preventing coagulation of the blood. Chicken blood containing EDTA or citrate does not clot but separates on storage or after centrifugation into a clear yellow plasma and a dark red suspension of erythrocytes and other cellular material. The plasma was decanted and saved; the erythrocyte suspension was discarded.

Two qualitative methods were used for the detection of antibodies in the chicken plasma. The ring or interfacial test involved the layering of antigen solution on antiserum and observing the interface by indirect illumination. Formation of a ring of precipitation at the interface was presumptive evidence of antibody activity. The second method was the agar gel diffusion procedure of Ouchterlony (36). Antigen solutions and plasma were placed in separate wells cut in agar gel films. The solutions diffused and formed visible lines or bands of precipitation where concentrations of antigen and antibody were optimal. Diffusion in agar gel prepared in 0.9% saline resulted in nonspecific precipitation around the plasma wells (7, cf. 6). In most diffusion tests, agar gel was prepared in 4% NaCl solution. The agar gel films were also washed after diffusion in 4% saline to remove unprecipitated serum proteins, then were dried and stained.

Blood samples were collected one week after each injection and examined for antibody activity. After tertiary and subsequent immunizations, about 20% of the chickens failed to produce antibodies. These chickens were discarded. Plasma obtained from chickens in response to each of the three immunizing antigens were pooled each week, respectively, and frozen at -20°C. When a sufficient-antiserum volume had been accumulated it was used in immunochemical experiments.

SYNTHESIS OF PHLOROGLUCINOL HAPTENS

The phloroglucinol azophenyl-β-D-glucoside, -xyloside, and -cellobioside were prepared by the procedure of Yariv, et al. (28). Some of the properties of these compounds are listed in Table II.

TABLE II

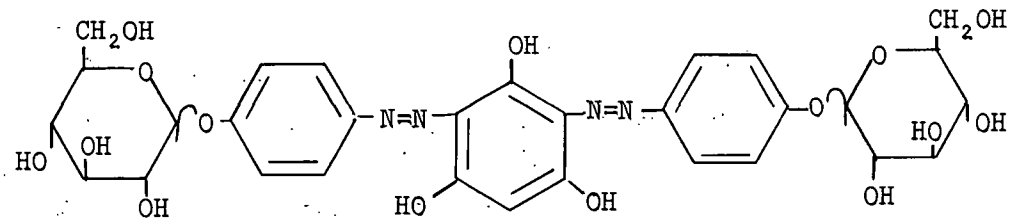
PROPERTIES OF PHLOROGLUCINOL HAPTENS

Hapten	Carbohydrate, %	D.S. (Lower Limit)	D.S.[a] (Upper Limit)	Extinction Coeff. at 398 nm.
Phloroglucinol azophenyl-β-D-glucopyranoside	51.4	1.85	2.58	390
Phloroglucinol azophenyl-β-D-xylopyranoside	46.3	1.75	2.53	413
Phloroglucinol azophenyl-β-D-cellobioside	69.6	1.75	2.52	272

[a]Most probable D.S.

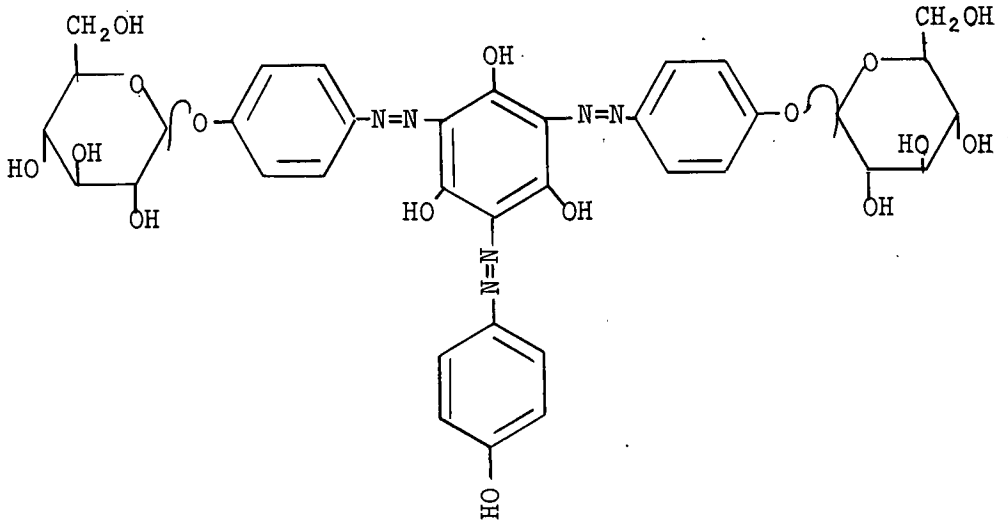
To be effective in precipitation reactions the phloroglucinol compounds must be at least difunctional in the glycosidic haptenic group. Any monosubstituted material acts as an inhibitor rather than as a precipitating agent (37). A phloroglucinol glucoside (phloroglucose) with D.S. 1.8 would have a maximum of 20% monosubstituted material. In terms of molar concentrations, however, there would be about 30 mole percent of the monoglucoside compound present in the mixture. Phloroglucose with D.S. 2.5 would have very little monosubstituted material.

Two D.S. values are given for these compounds. The lower values assume that only azophenyl-glycoside groups couple to the phloroglucinol. The higher D.S. values assume that the phloroglucinol is trisubstituted with azophenol groups, most of which still have the glycoside attached. These two types of compounds are illustrated in Fig. 1.



Phloroglucinol Diazophenyl-β-D-Glucoside

Glucoside D.S. - 2.00 52.2% Glucose



Phloroglucinol Triazophenol-di-β-D-Glucoside

Glucoside D.S. - 2.00 44.4% Glucose

Figure 1. Structures of Phloroglucinol Compounds

It is seen that both compounds are difunctional in glucose. The trisubstituted compound has a much lower percent glucose due to the increased weight of the additional azophenol group.

Several attempts were made to separate the probable mixtures of mono-, di-, and trisubstituted compounds making up the phloroglucinol haptens. Thin-layer and paper chromatography using several chromatographic systems failed to produce any clear separations. In most cases there was only a streaking of the original sample spotted. Phloroglucinol-cellobioside (phlorocello) was passed through a Bio-Gel P-2 column. Bio-Gel P-2 is a polyacrylamide molecular sieve with an exclusion limit of 2600 molecular weight. The phlorocello passed through as a single narrow band. All the phloroglucinol haptens were examined by the disk electrophoresis technique of Davis (31). Again there were no detectable band formations indicative of discrete chemical configurations.

The molar extinction coefficients listed in Table II were determined by measuring the absorption of the polyvalent haptens at 398 nm. in a 1-cm. cell at a concentration of 10 µg./ml. There was an absorption maximum at this wavelength for all of the phloroglucinol compounds. Yariv, et al. (28) found an extinction coefficient of 405 for their phloroglucose. The phloroglucose and phlorocello prepared by Gleich and Allen (29) had extinction coefficients of 416 and 278, respectively, at the same wavelength. Thus, the extinction coefficients of the phloroglucinol haptens produced in this study were comparable to those obtained by previous workers.

Yariv, et al. (28) synthesized a phloroglucinol azophenyl-glucoside, galactoside, and lactoside. Only the phlorogalactose was completely substituted with azophenyl-galactoside groups. Hydrolysis of their glucoside and lactoside compounds produced pure phloroglucinol triazophenol. The phloroglucinol was then trisubstituted in the coupling with the diazo salt but some of the glycosidic groups were hydrolyzed

during the course of the reaction. Since the extinction coefficients at 398 nm. for the compounds produced in this study were comparable to those found by Yariv, et al. (28) it is likely that these compounds were similarly trisubstituted and that the higher D.S. values in Table II were valid.

PLASMA FRACTIONATION AND ABSORPTION

Some plasma pools were fractionated by the ammonium sulfate precipitation technique of Campbell, et al. (32). While this method was supposed to result in pure gamma globulin fractions, it was found by disk electrophoresis that beta globulins were also present.

Two plasma samples were fractionated on a Bio-Gel P-200 column. The column, 5-cm. inside diameter and 62-cm. packed height, was eluted with phosphate buffer, pH 7.2. Since the exclusion limit of P-200 is 200,000 molecular weight, the macroglobulins (750-800,000 mol. wt.) were excluded and separated from the slightly retarded 7S globulins (150-160,000 mol. wt.).

Absorption is defined as the removal of antibody from an antiserum by a precipitatable antigen. Most of the plasma pools were absorbed with BSA to remove antibodies specific for this carrier protein. In a typical absorption 5 mg. of BSA in physiological saline solution were added to 250 ml. of pooled plasma. After 1 hour at room temperature the turbid suspension was stored at 4°C. until a precipitate formed, usually overnight. Centrifugation and decantation separated the sorbed plasma from the BSA-antibody precipitate. The process was repeated 3 or 4 times with 1 mg. additions of BSA until the plasma was completely absorbed.

QUANTITATIVE PRECIPITIN AND HAPTEN INHIBITION STUDIES

The detailed procedures used for quantitative precipitin and hapten inhibition studies are given in Appendix II. Precipitin tests were made with all of the

clarified plasma pools before and after absorption, using all of the immunizing antigens. The specific precipitates obtained were analyzed for nitrogen content by a Folin-Ciocalteu method (Appendix III).

Precipitin tests were also made with all of the phloroglucinol haptens. Since the phloroglucinol compounds reacted with the Folin-Ciocalteu colorimetric reagent, a different procedure was required for the determination of precipitate nitrogen. The procedure used was essentially the same as that described by Yariv, et al. (28) and by Gleich and Allen (29). The details of this procedure are given in Appendix IV.

Quantitative hapten inhibition studies were made with unabsorbed anticellobiose-BSA plasma using phlorocello as the precipitating antigen.

HAPTENS

The monosaccharides, monosaccharide derivatives, and some common disaccharides were all commercially available.

A sample of laminaribiose (3-O-β-D-glucopyranosyl-D-glucose) was a gift from Peter Z. Allen at the School of Medicine and Dentistry, University of Rochester, Rochester, New York.

RESULTS AND DISCUSSION

AGAR GEL DIFFUSION

Double diffusion of plasma and antigens in agar gel films prepared on microscope slides was used for the qualitative detection of antibodies. Figure 2 illustrates five sets of typical double diffusion experiments. The accompanying sketch indicates the antigens and plasma used in the diffusion tests and the author's interpretation of the significant precipitation patterns.

The first set (Fig. 2) illustrates the pattern of precipitation bands observed from diffusion of antiglucose-BSA* plasma against all of the immunizing antigens and BSA. Broad, diffuse precipitation bands were observed with all the antigens making it difficult to detect convergence or spurring.

The second set (Fig. 2) illustrates the effect of concentration on precipitate band formation. At the highest antigen concentration used, the band is broad and close to the center well containing immune plasma. Band thickness decreased and distance of the band from the center well increased as the concentration of the antigen solution in the outer wells decreased. All of the precipitation bands were convergent.

The third set (Fig. 2) illustrates the response of antiglucose-BSA plasma against the immunizing antigens and BSA. There were three distinct bands against glucose-BSA and xylose-BSA, two bands against cellobiose-BSA, and one sharp band against BSA.

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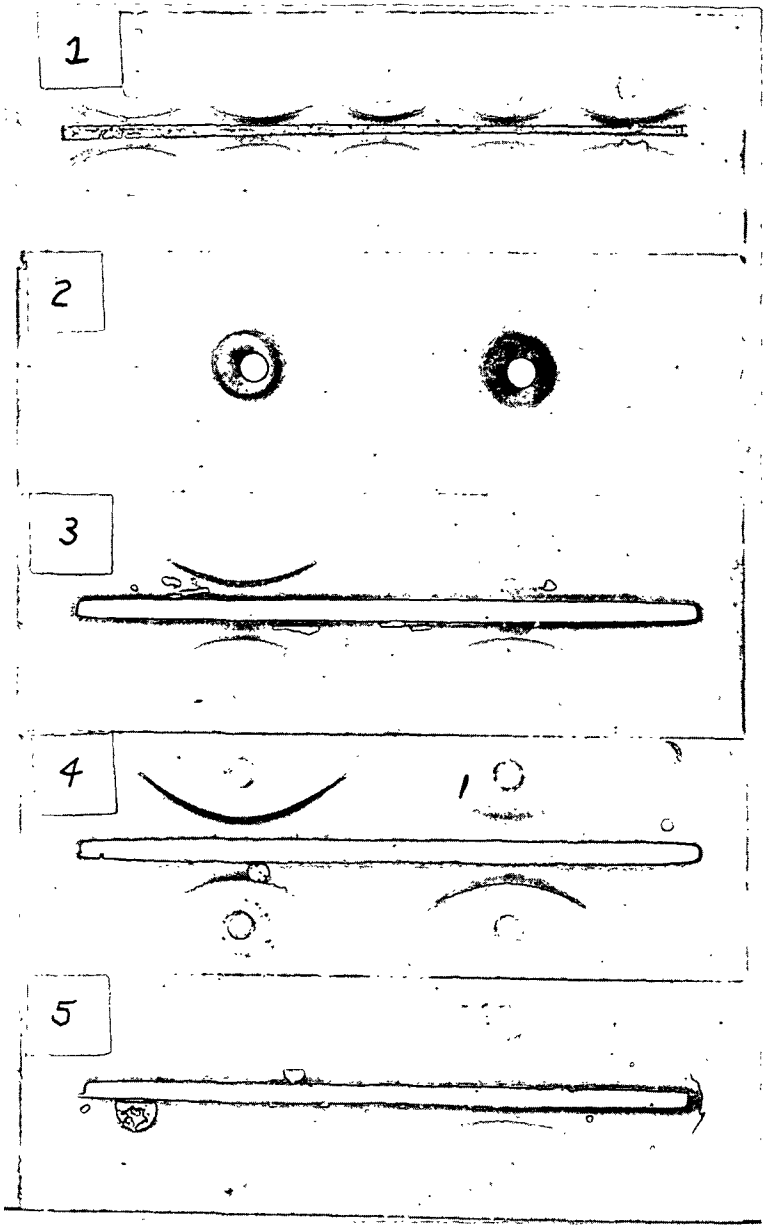
*BSA-p-phenylazo-β-D-glucopyranoside was designated as glucose-BSA. Immune plasma obtained against this antigen was designated as antiglucose-BSA plasma. Analogous designations were used for the other two immunizing antigens.



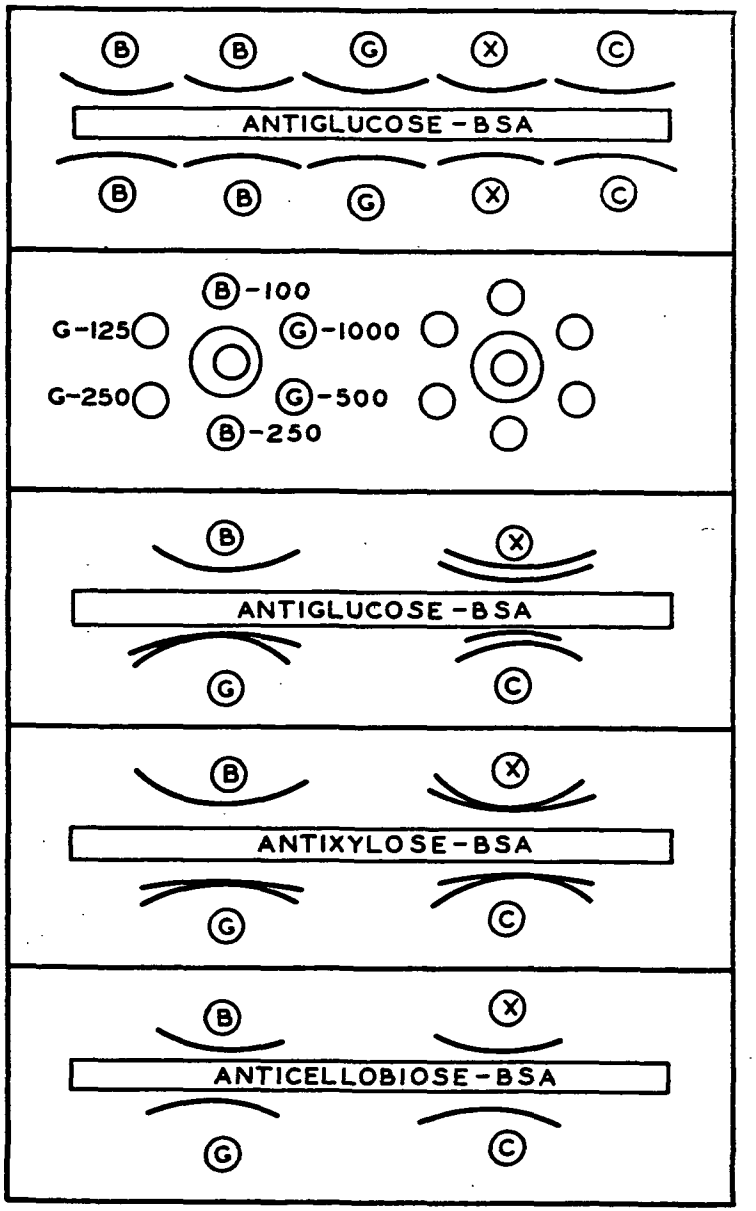


Figure 2. Agar Gel Diffusion Tests: Stained Slides. Key: B = BSA, G = Glucose-BSA, X = Xylose-BSA, C = Cellobiose-BSA. In Slide 2: B-100 = Concn. of BSA, 100 µg./ml.; Analogous Designations for Glucose-BSA, both Center Wells Contained Antiglucose-BSA

Antixylose-BSA plasma diffusion is shown in the fourth set (Fig. 2). There were two distinct precipitation bands against xylose-BSA, glucose-BSA, and cellobiose-BSA but again only one sharp band against BSA.

The anticellobiose-BSA diffusion illustrated in set five (Fig. 2) shows a distinct precipitation band only with the homologous antigen, cellobiose-BSA. Close inspection of the slide reveals light, diffuse bands of precipitation against all the other antigens and BSA.

The number of precipitation bands indicates the minimum number of reactive species of antibodies present, assuming no artifacts due to temperature and concentration (32). Two species with the same rate of diffusion in the agar gel would be observed as a single band when precipitated. BSA contained about equal amounts of albumin and prealbumin when examined by disk electrophoresis but produced only a single band of precipitation.

The multiple bands observed in Sets Three and Four could be the result of antibodies specific for different determinant groups in the immunizing antigens or to different molecular weights of antibodies specific for the same determinant group. Multiple banding of the antiglucose-BSA and antixylose-BSA plasmas against immunizing antigens was not always observed. This was an indication that precipitating macroglobulin antibodies were not present. A more detailed analysis of agar gel diffusion tests was not made since these tests were used primarily to monitor antibody production in individual chickens.

PLASMA FRACTIONATION

Figure 3 shows the results of fractionation of a pooled anticellobiose-BSA plasma sample on a Bio-Gel P-200 column. The ultraviolet absorption of the effluent

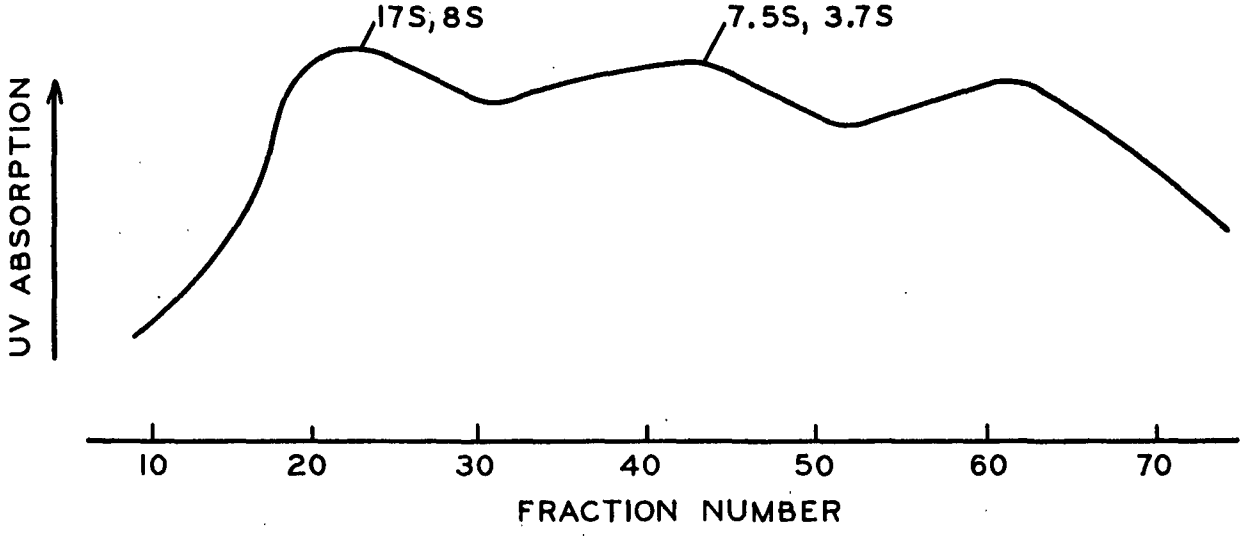


Figure 3. Bio-Gel P-200 Fractionation of Anticellobiose-BSA Plasma. Sedimentation Coefficients of Selected Fractions

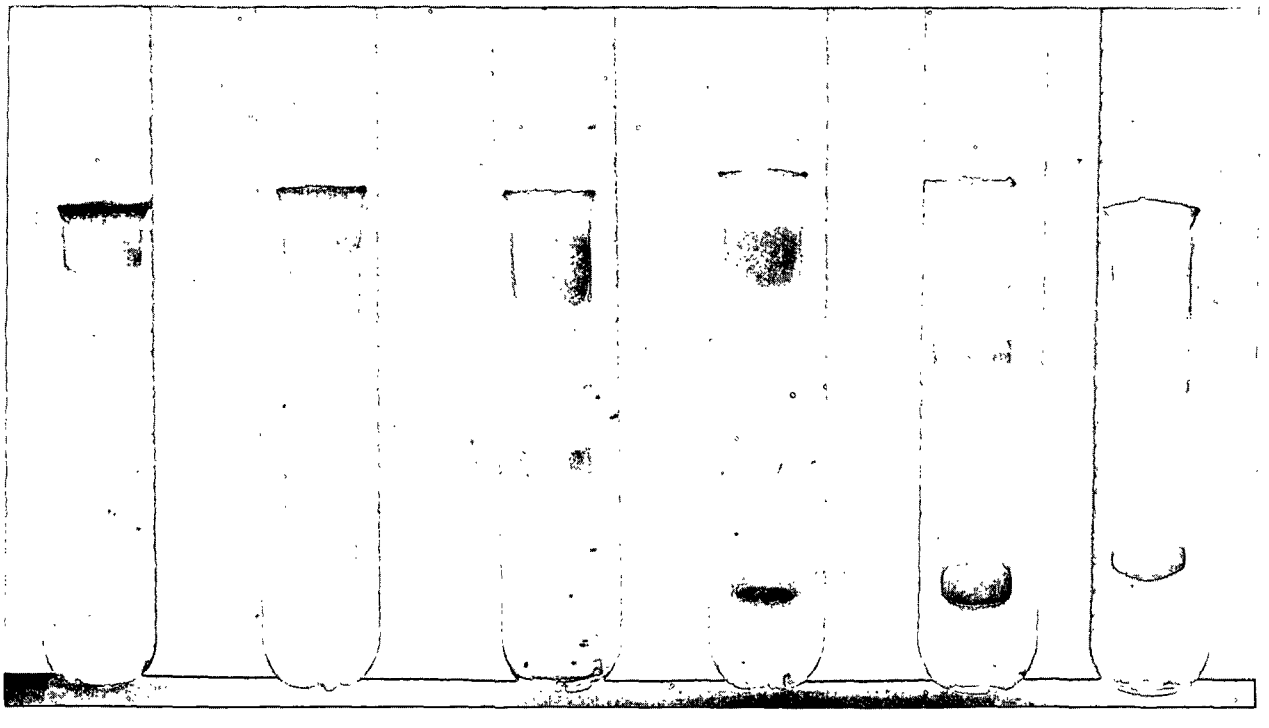


Figure 4. Disk Electrophoresis Gels of Selected Fractions (Fig. 3)

curve had three peaks and was similar in appearance to fractionations made by Říha (14) with a Sephadex G-200 column.

Samples from the fractions collected were examined by disk electrophoresis and by ultracentrifugation (Spinco, Model E Ultracentrifuge). The sedimentation coefficients determined are indicated in Fig. 3. Figure 4 is a photograph of the stained disk electrophoresis gels of selected fractions.

The mobilities of the components of the first elution peak were similar to those of gamma and epsilon globulins. There were two peaks in the ultracentrifuge with 17S and 8S sedimentation coefficients. Significantly there were no 19S or 21S components present, sedimentation coefficients associated with macroglobulin antibodies.

The second elution peak contained primarily gamma globulins but β_2 and α globulins were also observed in the electrophoresis gels. Initially, only a single peak was observed in the analytical ultracentrifuge with 7.5S mobility. With longer centrifugation time a smaller trailing peak with 3.7S sedimentation coefficient was observed.

The third peak consisted of albumin and the remaining β and α globulins. The expected transition of plasma proteins in the intervals between the major peaks were found by disk electrophoresis of samples collected in these regions. Between the first and second peak there were diminishing amounts of ε globulins and increasing amounts of γ and β globulins. The interval between the second and third peaks contained decreasing gamma globulins and the appearance of albumin.

Figure 5 illustrates the results of fractionation of a pooled antiglucose-BSA plasma sample on a Bio-Gel P-200 column. The ultraviolet (UV) absorption of the column effluent exhibited the expected three peaks. Aliquots of fractions

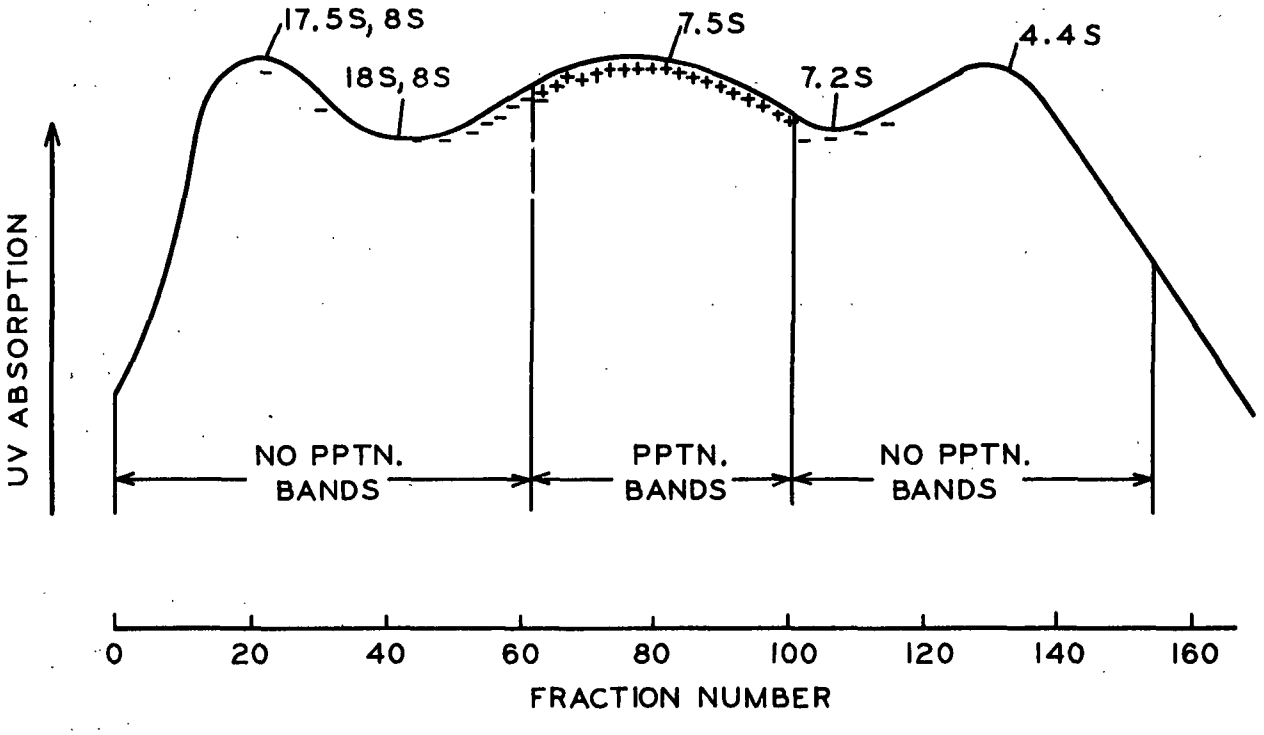


Figure 5. Bio-Gel P-200 Fractionation of Antiglucose-BSA Plasma. Sedimentation Coefficients of Selected Fractions, Above Elution Curve. Results of Interfacial Tests, Below Elution Curve. Results of Gel Diffusion Tests, Along Fraction Number Axis

corresponding to the peaks and minima of the UV trace were examined in the analytical ultracentrifuge. The sedimentation coefficients determined for the various components are also given.

Three fractions in the first peak were examined in the ultracentrifuge. Two components were observed in each of the fractions with sedimentation coefficients ranging from 17.0 to 17.8S for the rapidly sedimenting component and 7.4 to 8.8S for the slower component. The average values for sedimentation coefficient were 17.5S and 8S. Again no 19S or 21S macroglobulins were present.

In the transition between the first and second peaks there were still two components with 18S and 8S sedimentation coefficients. Samples from the second peak, transition between the second and third peaks, and the third peak all resolved single components in the ultracentrifuge with sedimentation coefficients of 7.5S, 7.2S, and 4.4S, respectively.

Fractions were examined for antibody activity by both the ring or interfacial test and by diffusion against antigen in agar gel films. The results of both tests are given in Fig. 5. The minus signs along the UV trace indicate a negative ring test. The plus signs, indicative of a positive ring test, were found only in fractions from the second elution peak. The same results were observed in agar gel diffusion tests. There were no bands of precipitation with antigen for diffusion of fractions from the first and third elution peaks. Precipitation bands were observed only with fractions from the second peak. The results of ring and gel diffusion tests coincided. All fractions with a positive ring test produced precipitation bands against antigen in agar gel films.

In summary, both fractionations resolved 17S components, 7S globulins, and albumin. The high molecular weight, rapidly sedimenting components of the leading

elution peak contained no antibody activity by interfacial or gel diffusion tests. Antibody activity was associated only with the 7S, gamma globulins.

QUANTITATIVE PRECIPITIN STUDIES

Antiglucose-BSA Plasma

A pooled sample of antiglucose-BSA plasma was divided into three equal portions. One portion was frozen in a dry ice-acetone bath, lyophilized, and reconstituted to the same volume in distilled water. Another portion was fractionated by the ammonium sulfate precipitation technique of Campbell, et al. (32). The globulin precipitate from the fractionation was dialyzed against 0.9% saline solution and adjusted to its original volume. All three portions were used in 0.2-ml. amounts in quantitative precipitin tests with BSA, glucose-BSA, and xylose-BSA. Three equivalent levels of antigen addition were used. The results of these tests appear in Table III.

TABLE III

PRECIPITATION OF ANTIGLUCOSE-BSA PLASMA WITH IMMUNIZING ANTIGENS AND BSA

Antigen	Antigen N Added, µg.	Control N Pptd., µg.	Lyophilized N Pptd., µg.	Fractionated N Pptd., µg
BSA	1.4	16.0	16.6	8.6
	2.1	12.2	14.0	7.2
	2.8	9.5	10.8	4.6
Glucose-BSA	1.5	16.1	19.1	12.0
	2.2	17.8	20.2	13.3
	2.9	19.4	22.4	11.8
Xylose-BSA	1.2	13.0	13.7	8.5
	2.0	15.6	15.8	8.7
	2.6	17.4	15.8	10.2

Lyophilization had no adverse effect on antibody precipitation. Approximately equivalent amounts of antibody nitrogen were precipitated from control and lyophilized plasma. Fractionation reduced the amount of nitrogen precipitated by antigen.

In the standard precipitin test, the small amount of antigen added is completely precipitated by antibody. Addition of increasing amounts of antigen to constant amounts of antibody produces increased precipitation. When the optimum concentrations of antigen and antibody are reached, the amount of precipitate is at a maximum. This optimum concentration is the equivalent point. Beyond equivalence the extra antigen added produces soluble antigen-antibody complexes. The excess antigen inhibits the formation of insoluble precipitates and the amount of precipitate formed diminishes.

Precipitation of all three portions of plasma by BSA decreased with increasing amount of antigen added (Table III). The point of maximum precipitation was at 1.4 µg. BSA nitrogen (N) addition or less. The increasing amounts of BSA added were apparently in the antigen excess region, since the amount of precipitate decreased with increasing antigen concentration.

Precipitated nitrogen with lyophilized and control plasmas increased with increasing additions of glucose-BSA. Subtraction of antigen nitrogen from the total nitrogen precipitated still indicated a modest increasing trend. The incremental increase of precipitated nitrogen with antigen added was slight and suggests approach to equivalence.

Homologous antigen, glucose-BSA, produced slightly greater amounts of precipitation than BSA or xylose-BSA with all plasmas. But, since the equivalence points were unknown, it is not certain that there would be a greater response with glucose-BSA at its equivalence value over that of BSA or xylose-BSA at their respective equivalence points.

The results of additional precipitin tests made with a pooled antiglucose-BSA plasma sample are given in Fig. 6. In these tests 0.5-ml. volumes of plasma were used. Precipitate nitrogen was determined by the Nessler reaction (32).

Precipitate amount increased with increasing antigen addition for all of the immunizing antigens and BSA. Equivalence was not achieved with any of the antigen-antibody systems. However, at any given antigen addition, precipitation with homologous antigen, glucose-BSA, exceeded precipitation with BSA or with either of the other two immunizing antigens. This indicates a specificity of the antibody for the p-azophenyl-β-D-glucoside group.

The precipitation of xylose-BSA and cellobiose-BSA with antiglucose-BSA plasma were nearly coincident and lower in value than precipitation with glucose-BSA or BSA. Arakatsu, et al. (27) found in their work with rabbit antisera produced against an α-glucosyl determinant that an α-isomaltosyl-BSA was a more efficient precipitating agent than homologous antigen, α-glucosyl-BSA. With chicken plasma in response to a β-glucoside determinant there was no similar effect with cellobiose-BSA as the precipitating agent.

A pool of antiglucose-BSA plasma was absorbed repeatedly with BSA until no further precipitation was observed. The absorbed plasma was used in 1.0-ml. amounts in precipitin tests with glucose-BSA and BSA. The results of these tests are presented in Fig. 7.

Absorption considerably reduced the amount of precipitate obtained with glucose-BSA. Before absorption, Fig. 6, the antibody response was at least 150 μg. N/ml. plasma. After absorption the response diminished to about 26 μg. N/ml. plasma, Fig. 7.

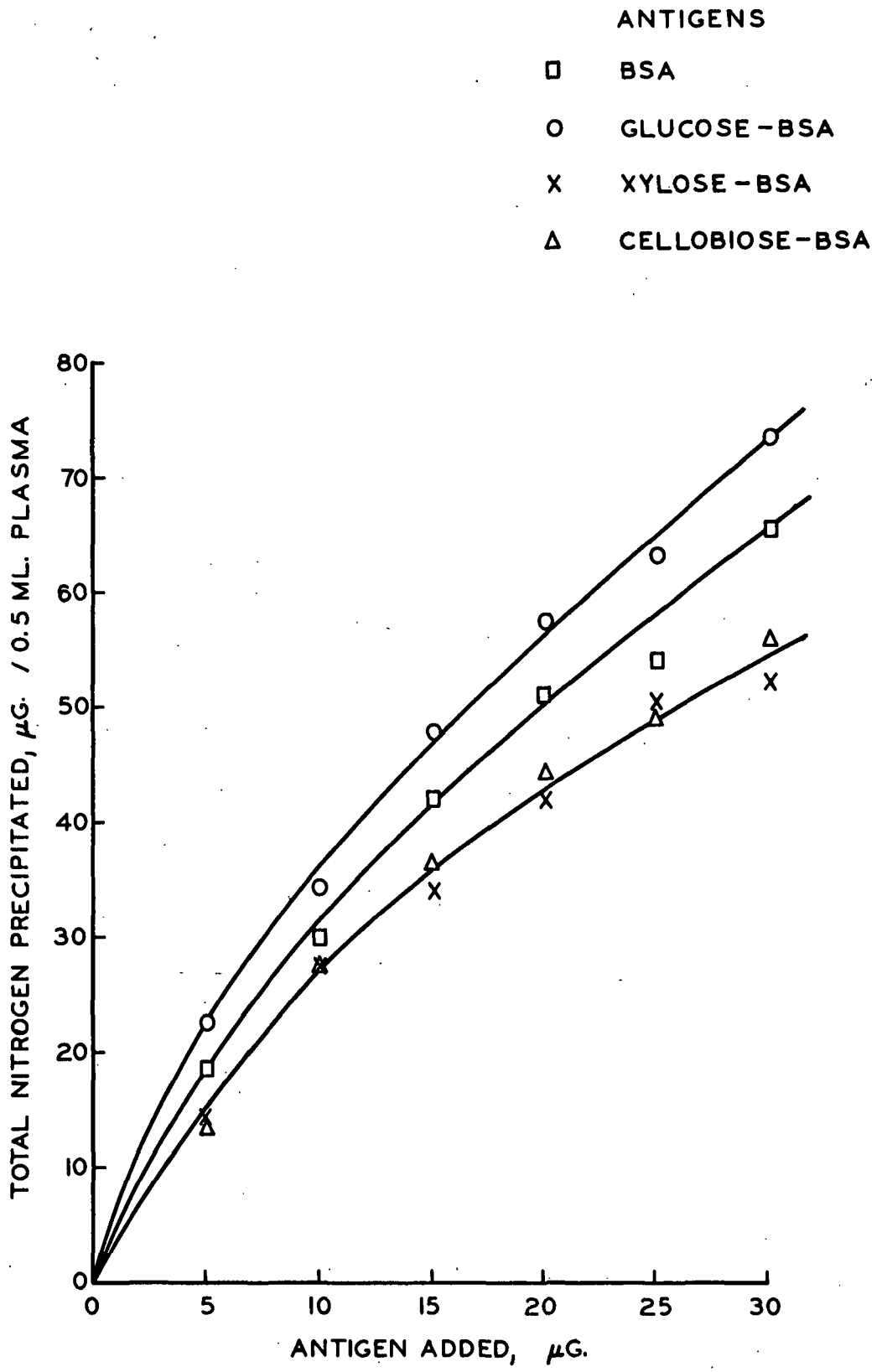


Figure 6. Quantitative Precipitin Tests: Antiglucose-BSA Plasma

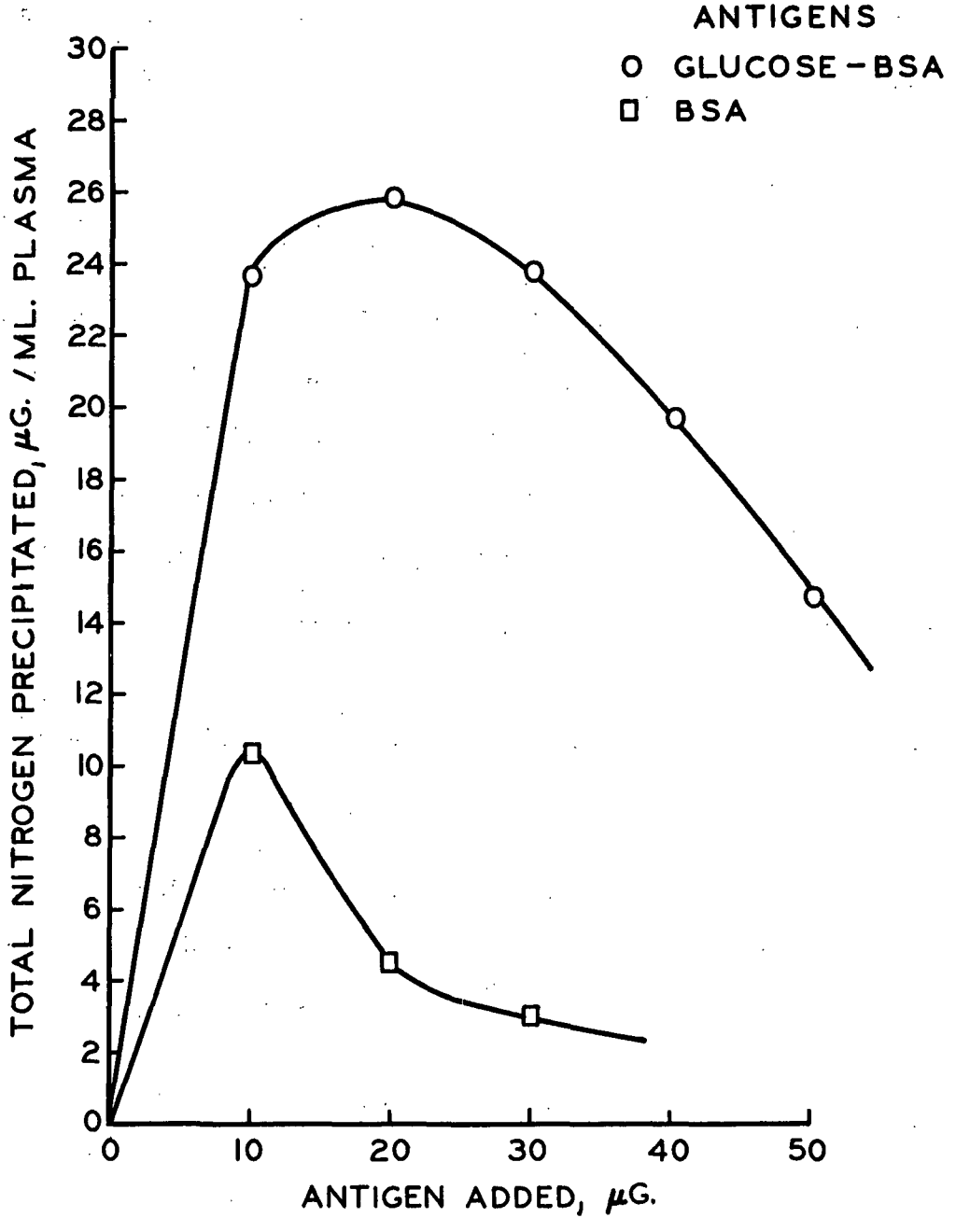


Figure 7. Quantitative Precipitin Tests: Partially Absorbed Antiglucose-BSA Plasma

It is apparent that absorption was incomplete, since there were at least 10 µg. of antibody nitrogen per ml. of plasma reactive with BSA. The response with glucose-BSA was greater than that with BSA, but again the equivalence point for BSA was uncertain, although the curve is drawn in Fig. 7 as if it were at 10 µg./ml. It could be on either side of that point but under 20 µg./ml. antigen added.

The plasma pool was absorbed with additional BSA and used again in precipitin tests with BSA and BSA conjugates. The results of these tests are given in Fig. 8.

This additional absorption further reduced the antibody response. With the exception of one data point, precipitation of the absorbed plasma with glucose-BSA was greater than precipitation with the other antigens and BSA. The one low point was probably due to an experimental error. The data were consistent with the concepts of specificity of the antibody for the p-azophenyl-β-D-glucoside determinant group. The magnitude of this antibody response was considerably lower than the response in rabbits to the same antigenic determinant. Yariv, et al. (28) found 210 µg. N/ml. in their rabbit antiserum to glucose-BSA specifically precipitated by phloroglucose.

There appeared to be slight residual reactivity for BSA despite the extensive absorption. A similar residual response for BSA was observed by Arakatsu, et al. (27) for several of their BSA absorbed rabbit antisera to an α-glucosyl-BSA.

Reactivity of the absorbed plasma with xylose-BSA and cellobiose-BSA was at the same low level of response found for BSA.

Several pools of antiglucose-BSA plasma were tested for reactivity with phloroglucose. The results of six separate tests are presented in Fig. 9.

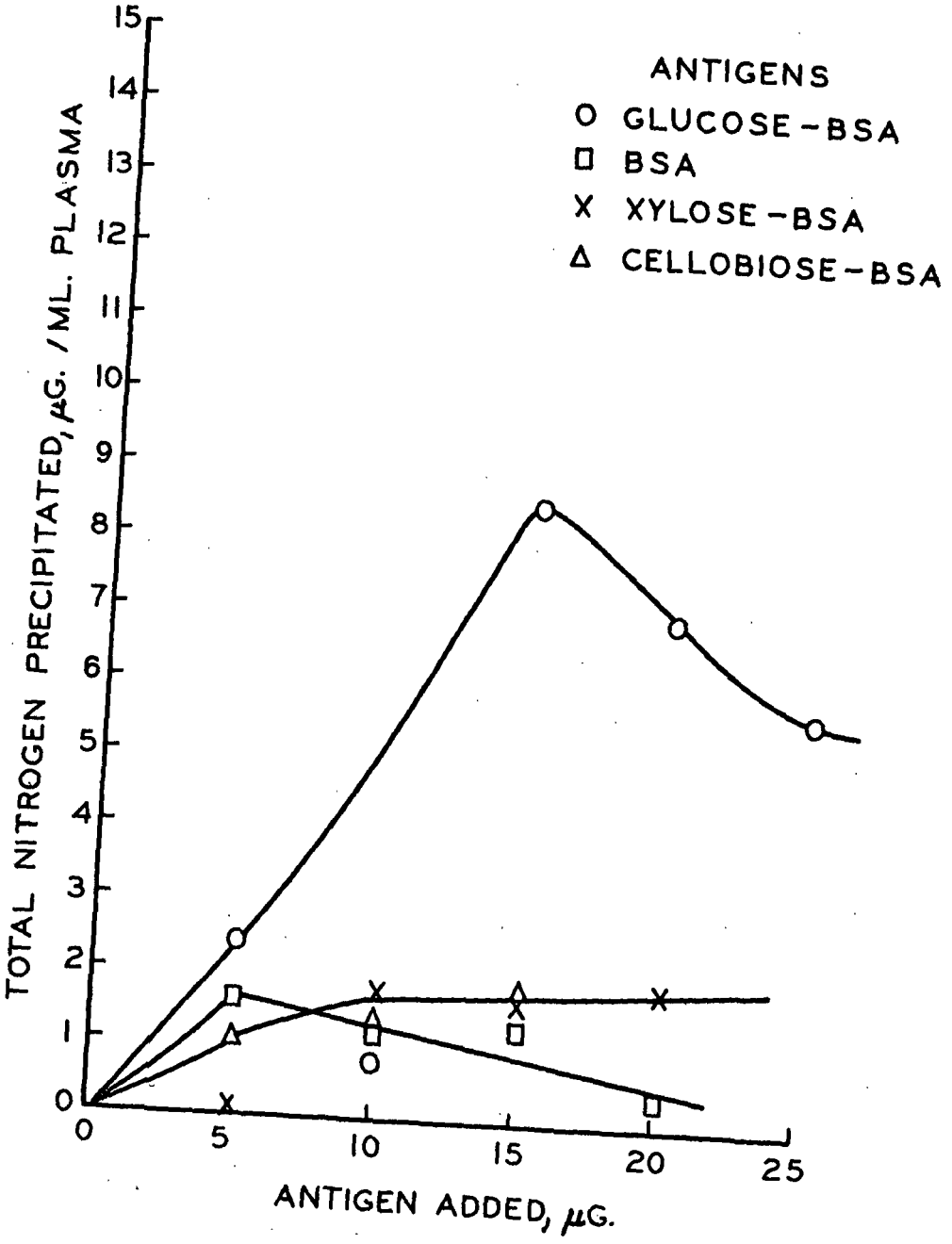


Figure 8. Quantitative Precipitin Tests: Absorbed Antiglucose-BSA Plasma

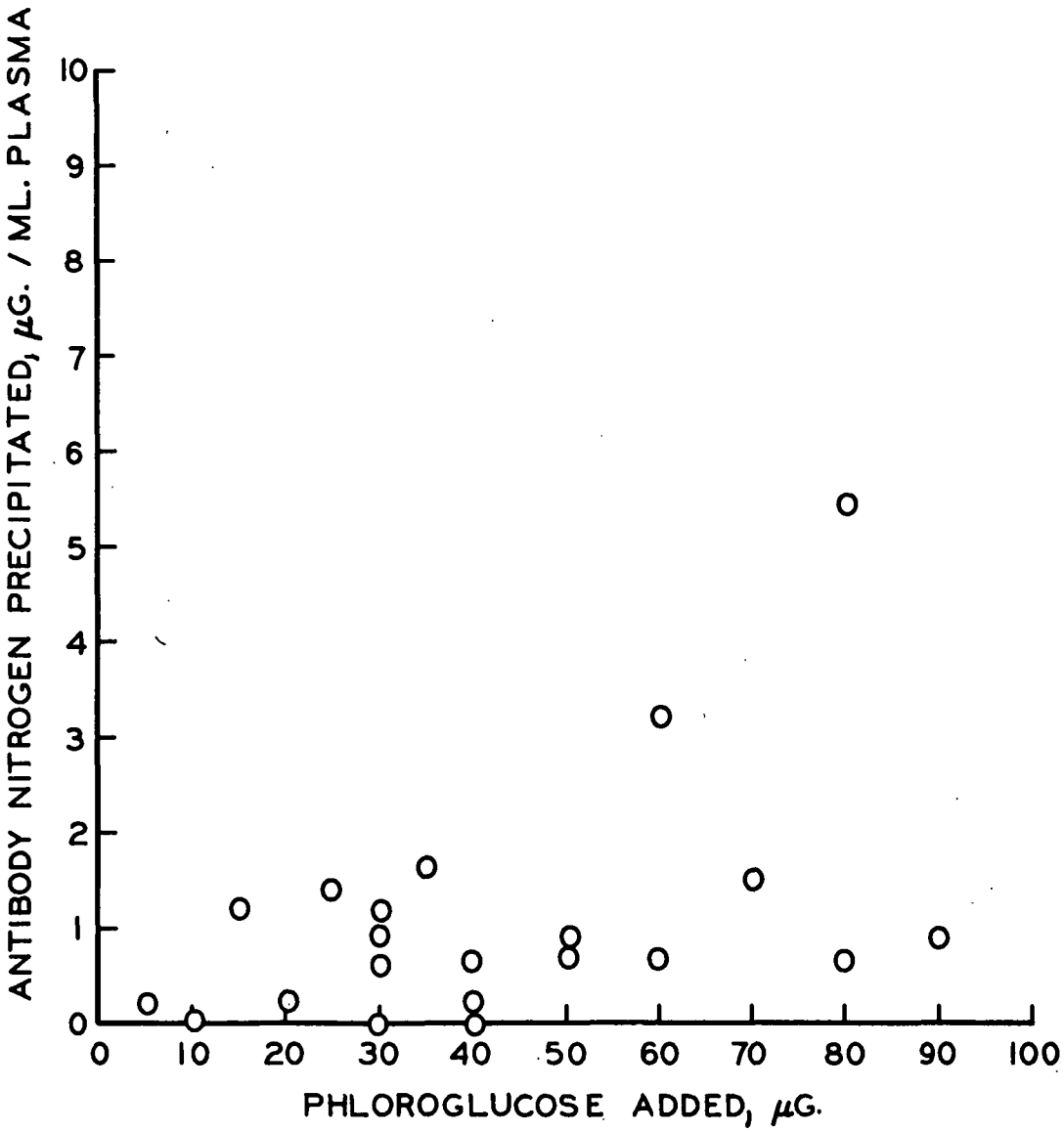


Figure 9. Quantitative Precipitin Tests: Antiglucose-BSA Plasma

With the exception of two data points all of the values for antibody nitrogen precipitated were on the order of 1 μg. The colorimetric method used to determine antibody nitrogen was only valid to ± 1 μg. N. The values then were not significantly different from zero.

Yariv, et al. (28) and Gleich and Allen (29) found that phloroglucose had a limited solubility. At 4°C., the temperature of the precipitin tests, the solubility of the phloroglucose used in this study was approximately 40 μg./ml. The two high data points were possibly due to a nonspecific coprecipitation of phloroglucose with plasma proteins.

The magnitude of the antibody response specific for the p-azophenyl-β-D-glucoside determinant from Fig. 8 was about 6-8 μg. N/ml. The same response was expected with phloroglucose. If phloroglucose contained monosubstituted material, however, the presence of these inhibitors would reduce the magnitude of the response. The lack of response with phloroglucose was unusual. It is shown subsequently that both of the other phlorohaptens were reactive in their respective antibody systems.

Antixylose-BSA Plasma

Plasma obtained in response to xylose-BSA was pooled and reacted with BSA and the other immunizing antigens in precipitin tests. The results of these tests are illustrated in Fig. 10.

The response of the unabsorbed plasma with homologous antigen was about 80 μg. N/ml. There was greater precipitation with xylose-BSA at its maximum value than there was with glucose-BSA or cellobiose-BSA at their maximum values. The equivalence point for BSA was again uncertain but under 20 μg. antigen added.

Equivalence with xylose-BSA occurred near 30 μg. addition. So and Goldstein (38) found in their studies with concanavilin A that in quantitative precipitin

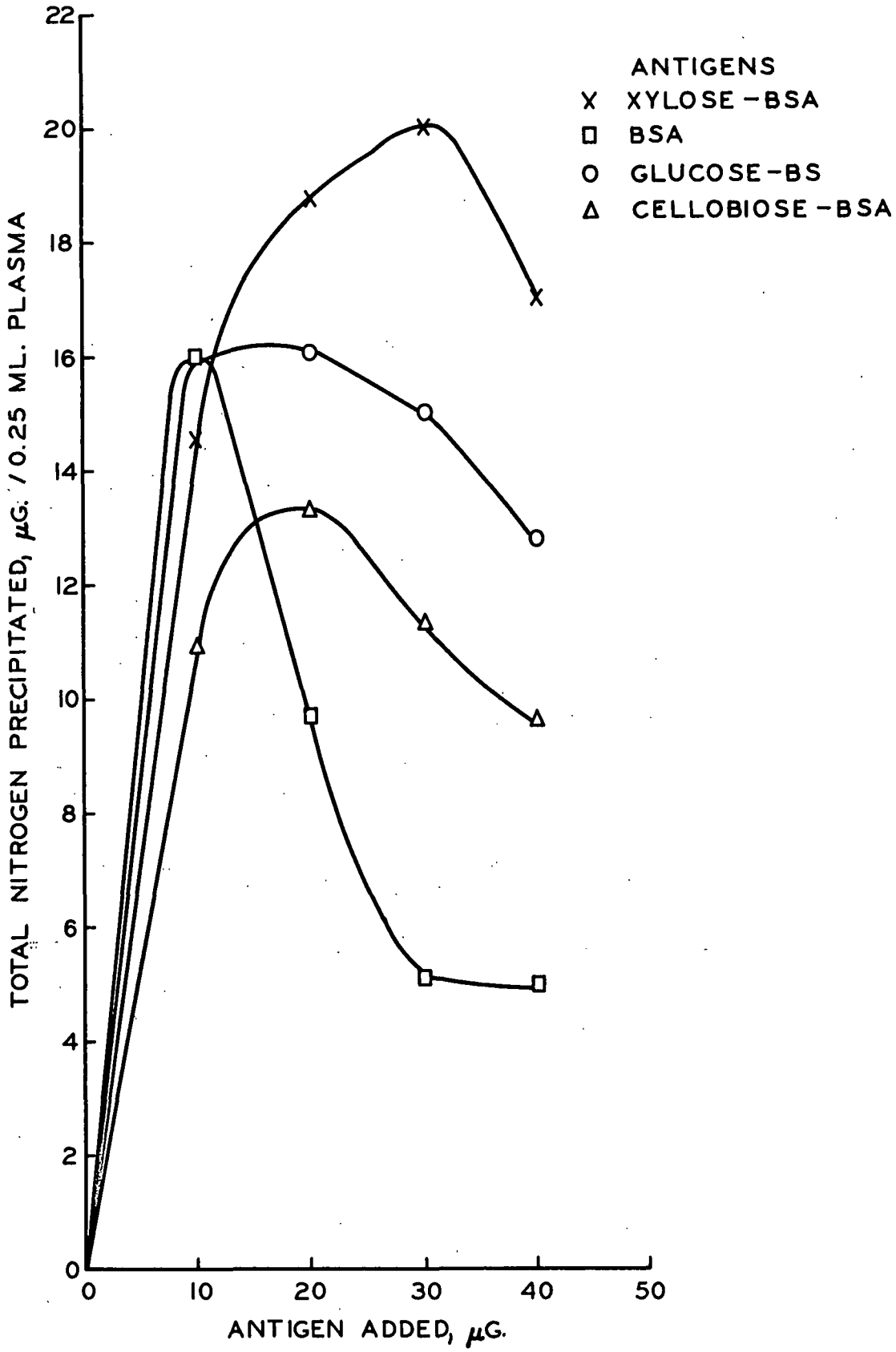


Figure 10. Quantitative Precipitin Tests: Antixylose-BSA Plasma

tests maximum precipitation was reached at lower levels of antigen addition for lower molecular weight precipitating antigens. Also, the lower molecular weight antigens were more effective in inhibiting precipitation in the antigen excess region. BSA had a lower molecular weight than any of the immunizing antigens. Inhibition of precipitation in the antigen excess region was greater with BSA than with the other BSA conjugates in the precipitin tests presented in Fig. 10.

Another antixylose-BSA plasma pool was absorbed repeatedly with BSA, fractionated by ammonium sulfate precipitation, and the gamma globulin fraction reconstituted to its original volume. This reconstituted gamma globulin fraction was used in precipitin tests with xylose-BSA to study the effects of aging, test duration, and pH. Figure 11 illustrates the results of these tests.

Absorption and fractionation reduced the response from 80 µg. N/ml. to about 25 µg. N/ml. Aging of the antiserum for three weeks at 2-4°C. had no effect on the precipitin curve. After five weeks of aging there was a noticeable decrease in precipitin values. Since no preservatives were added to the globulin solution, it was likely that antibody activity was lost due to bacterial action.

Precipitin tests were made with the five-week old gamma globulin fraction at pH 7.2 with test durations of 96 and 168 hours. There was no change in the precipitin curve for longer test periods. Increasing the pH of the precipitin test to 7.9 with this same aged gamma globulin fraction decreased the amount of precipitate formed.

All of the precipitin tests presented in Fig. 11 had maximum precipitation at 20 µg. additions of xylose-BSA. Neither aging nor a change in pH shifted the equivalence point.

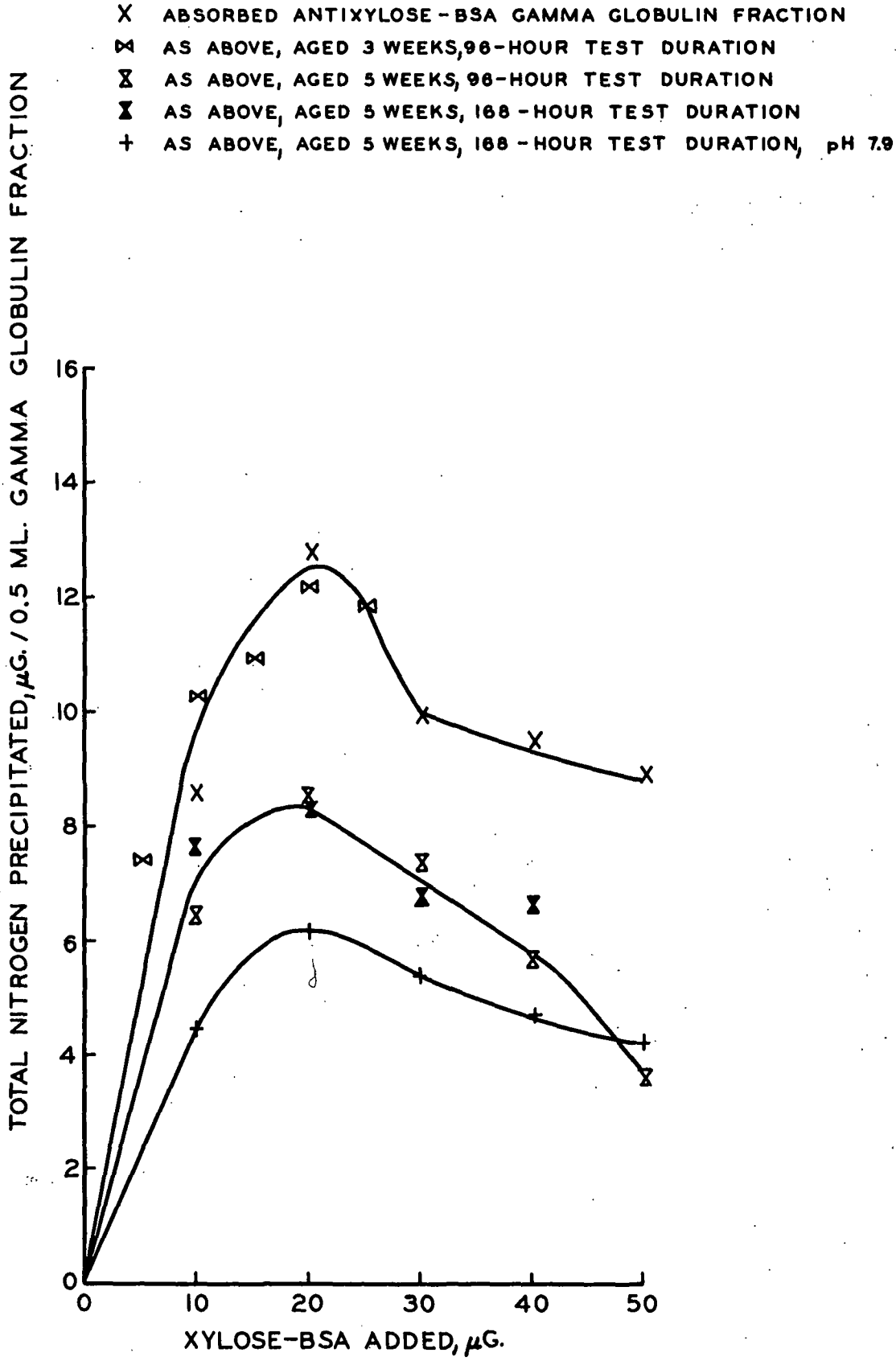


Figure 11. Quantitative Precipitin Tests: Antixylose-BSA Gamma Globulin Fraction

The remainder of this gamma globulin fraction was adjusted to 8% salinity and reacted with all of the immunizing antigens and BSA dissolved in buffered 8% saline solution. The results of these tests are given in Fig. 12.

The high salinity produced a pronounced increase in precipitated nitrogen and a shift in the equivalence point toward higher amounts of xylose-BSA added. Hersh and Benedict (15) found that chicken gamma globulins aggregate in 8% NaCl solution. The increased response, double that obtained in 0.9% saline, was probably due to the aggregation of normal globulins with the immune gamma globulins in the high salinity.

Since there was a residual response with BSA, the original plasma was not completely absorbed. Cellobiose-BSA and glucose-BSA both cross reacted with this gamma globulin fraction. Cross reactivity was due in part to the common protein substrate, BSA, but glucose-BSA was a better precipitating antigen than cellobiose-BSA. The structure of glucose-BSA should be more similar to xylose-BSA than to the structure of cellobiose-BSA. Homologous antigen, xylose-BSA, was the most effective precipitating antigen. This is in accord with specificity toward the p-azophenyl-β-D-xyloside determinant.

Another antixylose-BSA plasma pool was accumulated and completely absorbed with BSA. The results of quantitative precipitin tests made with this plasma pool are given in Fig. 13.

Both BSA and cellobiose-BSA exhibited a low residual response at all concentrations of antigen added. The same results were observed with absorbed antiglucose-BSA plasma.

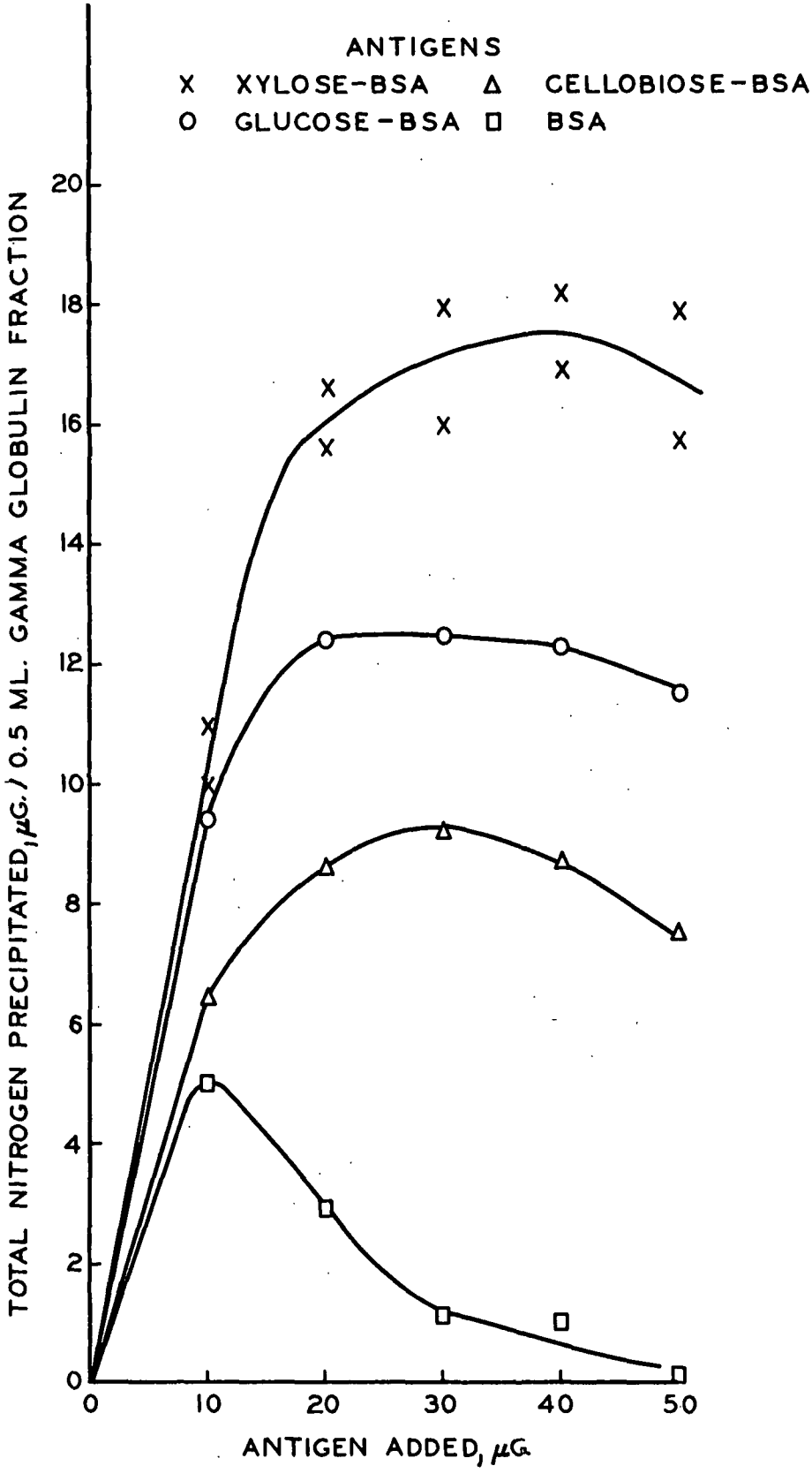


Figure 12. Quantitative Precipitin Tests: 8% Saline, Absorbed Antixylose-BSA Gamma Globulin Fraction

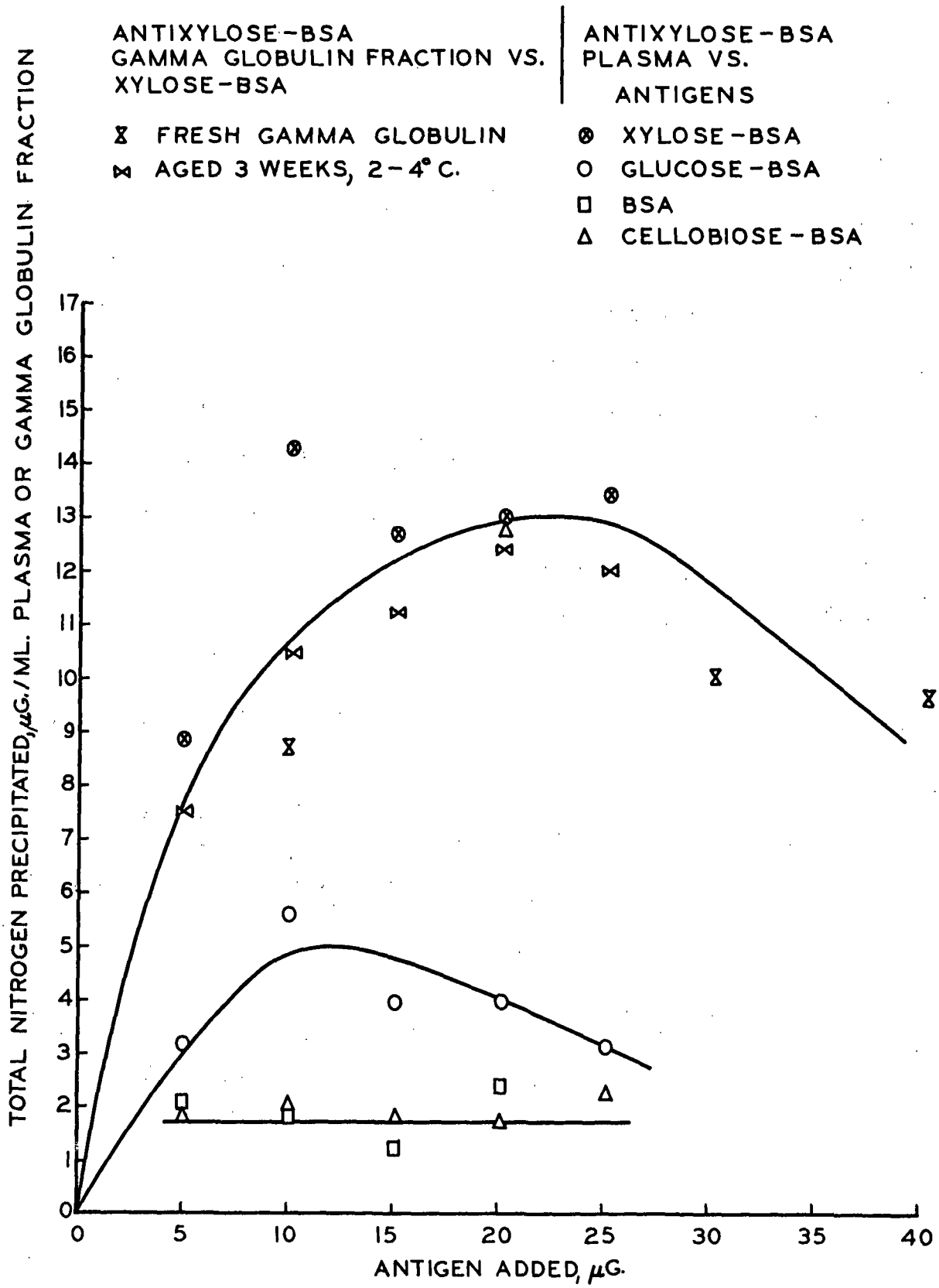


Figure 13. Quantitative Precipitin Tests: Absorbed Antixylose-BSA Plasma

Specificity for the p-azophenyl-β-D-xyloside group was demonstrated by the response obtained with xylose-BSA. Although the magnitude of this response was low, it was double that obtained in the glucose system.

There was cross reactivity of glucose-BSA with the absorbed antixylose-BSA plasma. The magnitude of this cross reaction was low but significant. All of the data points for precipitation with glucose-BSA were consistently greater than the residual response found for BSA and cellobiose-BSA. Some cross reactivity was expected since the configurations of glucose and xylose at carbon atoms one to four are identical. The increased response with xylose-BSA over that with glucose-BSA suggests that the xylose determinant conferred immunological specificity. That is, the absence of the hydroxymethyl group ($-CH_2OH$) at carbon five in xylose compared with the structure of glucose was a significant alteration in configuration and conferred antibody specificity.

Figure 14 presents the results of four precipitin tests made with absorbed antixylose-BSA plasma and phloroxylose. For these tests a phloroxylose solution was prepared at a concentration of 100 µg./ml. by dissolving the hapten in heated phosphate buffered saline solution. Precipitin tests were set up with this concentrated solution at room temperature but were incubated at 4°C. It was found that the concentration of the phloroxylose solution decreased upon standing for several days at room temperature to approximately 32 µg./ml. At the temperature of the precipitin test, 4°C., the saturation concentration was only 10 µg./ml. There was then a spontaneous precipitation of phloroxylose in the precipitin tests where additions of this phlorohapten resulted in concentrations greater than 10 µg./ml.

In precipitin tests with the phlorohaptens it was possible to determine the amount of antigen in the precipitates. Table IV presents the results of three

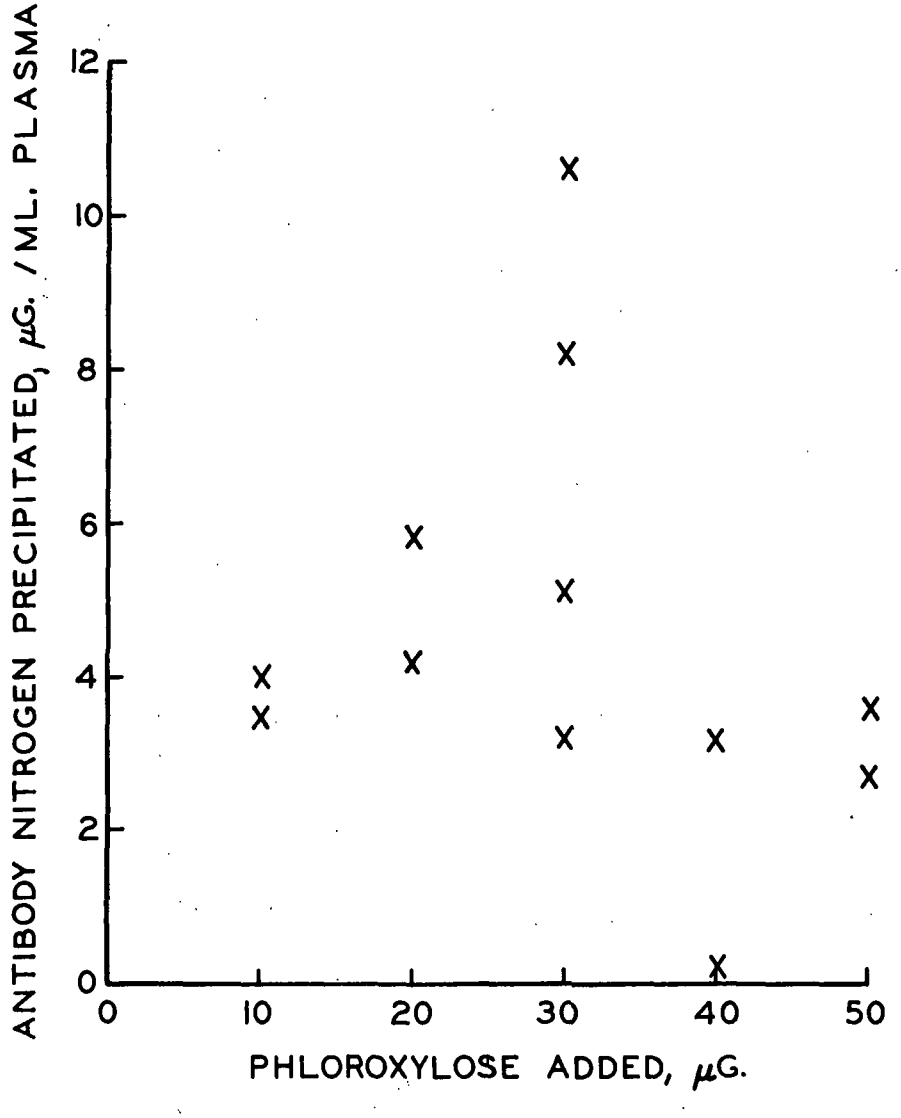


Figure 14. Precipitin Tests with Phloroxylose Hapten: Absorbed Antixylose-BSA Plasma

precipitin tests where phloroxylose hapten was used as the precipitating agent with antixylose-BSA, antiglucose-BSA, and anticellobiose-BSA plasmas.

TABLE IV

PRECIPITIN TESTS WITH PHLOROXYLOSE

Plasma[a]	Phloroxylose Added, µg.	Antibody Nitrogen, µg.	Phloroxylose in Ppt., µg.
Antixylose-BSA (absorbed)	10	3.5	2.2
	20	4.2	4.1
	30	8.2	6.1
	40	3.2	8.8
	50	2.7	15.8
Antiglucose-BSA (unabsorbed)	10	0.7	1.9
	20	0.0	3.1
	30	0.0	6.7
	40	0.0	11.1
	50	0.0	23.5
Anticellobiose-BSA (unabsorbed)	10	0.0	2.9
	20	0.0	4.7
	30	0.0	9.2
	40	0.0	12.9

[a] 1.0 ml. used in each test.

In each test the amount of antigen which was insoluble at each level of addition was about the same. Only in the test with absorbed antixylose-BSA plasma was there precipitation greater than that due to the antigen alone. The antibody response with this plasma was not a coprecipitation of plasma proteins with the phlorohapten since no similar response was observed with the other two plasmas. There was then a specific combination of antibodies with the phlorohapten.

A precipitin test was made with varying amounts of unabsorbed antixylose-BSA plasma and a constant amount of phloroxylose hapten, 10 µg. The results of this test are given in Table V.

TABLE V

PRECIPITIN TESTS WITH CONSTANT PHLOROXYLOSE

Plasma Added, ml.	Phloroxylose Added, µg.	Antibody Nitrogen, µg.	Phloroxylose in Ppt., µg.
0.125	10	6.2	1.4
0.25	10	6.2	1.6
0.50	10	7.8	3.9
1.0	10	7.8	3.8

With only 10 µg. of phloroxylose present there was no nonspecific precipitation of this hapten. Maximum observed antibody precipitation occurred at 0.5-ml. plasma addition. Half this amount of plasma reduced slightly antibody nitrogen precipitated while double this amount of plasma precipitated the same amount of antibody and phlorohapten. In 1.0 ml. of plasma there was excess antibody yet there was incomplete precipitation of the phlorohapten. Only about 4 µg. of the 10 µg. added were recovered in the specific precipitate. This was consistent with possible inhibition by monosubstituted material in the phloroxylose. However, Pauling, et al. (39) in their studies with several polyvalent haptens also did not obtain complete precipitation of hapten with excess antibody. The phlorohaptens, unlike protein antigens, apparently do not precipitate completely with excess antibody.

Anticellobiose-BSA Plasma

A pooled sample of anticellobiose-BSA plasma was reacted with the three immunizing antigens and BSA. The results of these tests are given in Fig. 15.

The antibody response was very low. Even with the homologous antigen the response with this unabsorbed plasma was only about 50 µg. N/ml. There was a definite specificity for the p-azophenyl-β-D-cellobioside group since precipitation with cellobiose-BSA at its equivalence point exceeded precipitation with BSA at its maximum point. The magnitude of this difference was about 12 µg. N/ml.

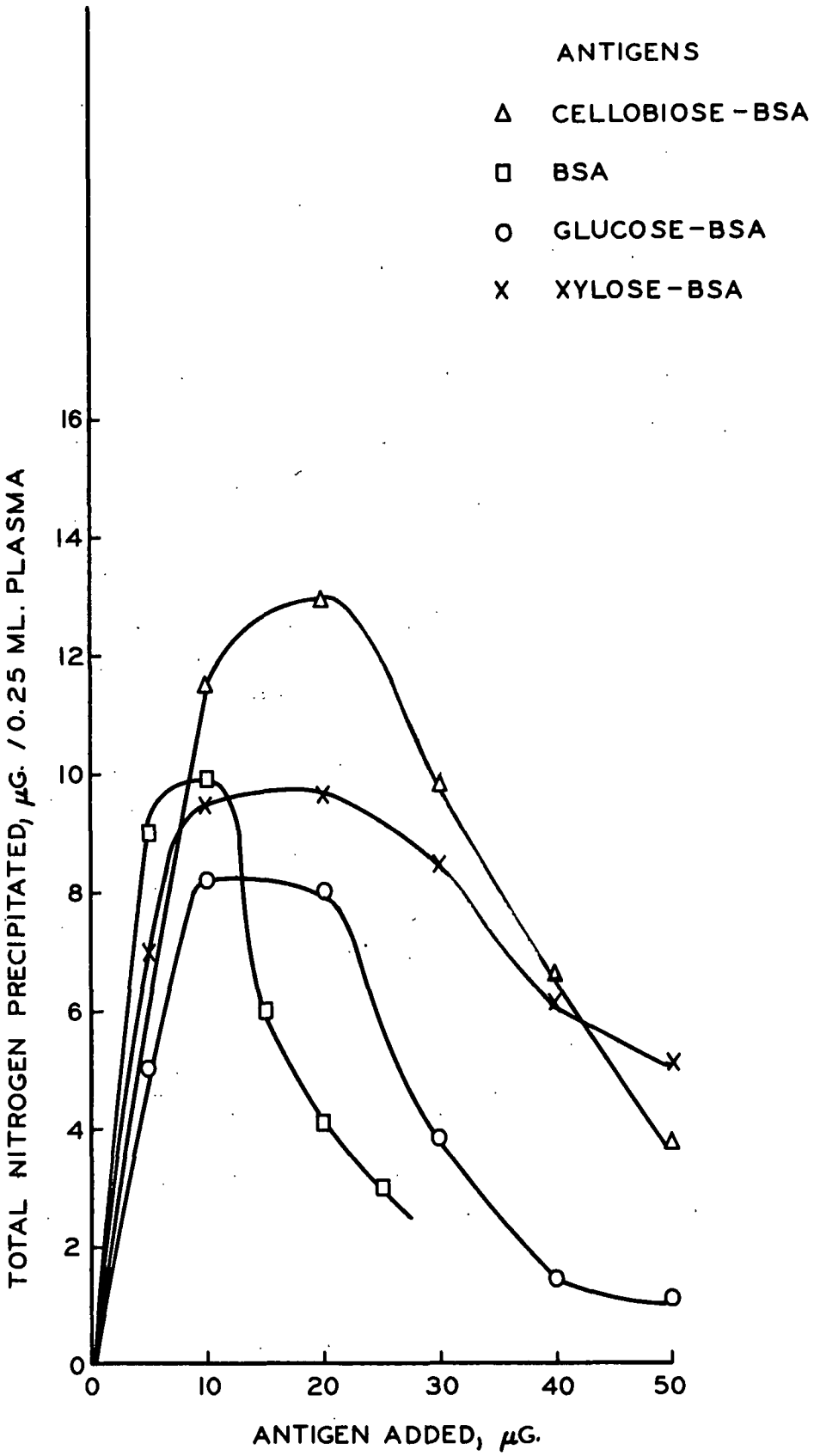


Figure 15. Quantitative Precipitin Tests: Anti-cellobiose-BSA Plasma

The equivalence point with BSA was reached at a lower level of antigen addition than that required for equivalence with the other antigens. Excess BSA produced considerable inhibition of precipitation. A similar effect was found by So and Goldstein (38) with the concanavilin A system.

Glucose-BSA was the least effective precipitating antigen in these tests. Maximum precipitation with xylose-BSA was about the same as that with BSA.

Another pooled sample of anticellobiose-BSA plasma was absorbed repeatedly with BSA. Precipitin tests made with this absorbed plasma and all of the antigens are illustrated in Fig. 16.

Absorption of the plasma was complete since there was no longer any reactivity with BSA. The response with homologous antigen, cellobiose-BSA, was at least 14 µg. N/ml. Both glucose-BSA and xylose-BSA exhibited the same extent of cross reactivity with this absorbed plasma. The cross reactivity was due to the presence of the p-azophenyl-glycoside groups since reactivity for the carrier protein, BSA, was removed by absorption.

A further demonstration of p-azophenyl-β-D-cellobioside specificity was found in precipitin tests made with anticellobiose-BSA plasma and the phlorohaptens. The results of these tests are given in Fig. 17.

There were about 14 µg./ml. of antibody nitrogen precipitatable by phlorocello. Gleich and Allen (29) with rabbit antisera to cellobiose-BSA found maximum responses of 100-160 µg. N/ml. precipitatable by phlorocello. The antibody response with chickens was then much lower than that found in rabbits for the same antigenic determinant.

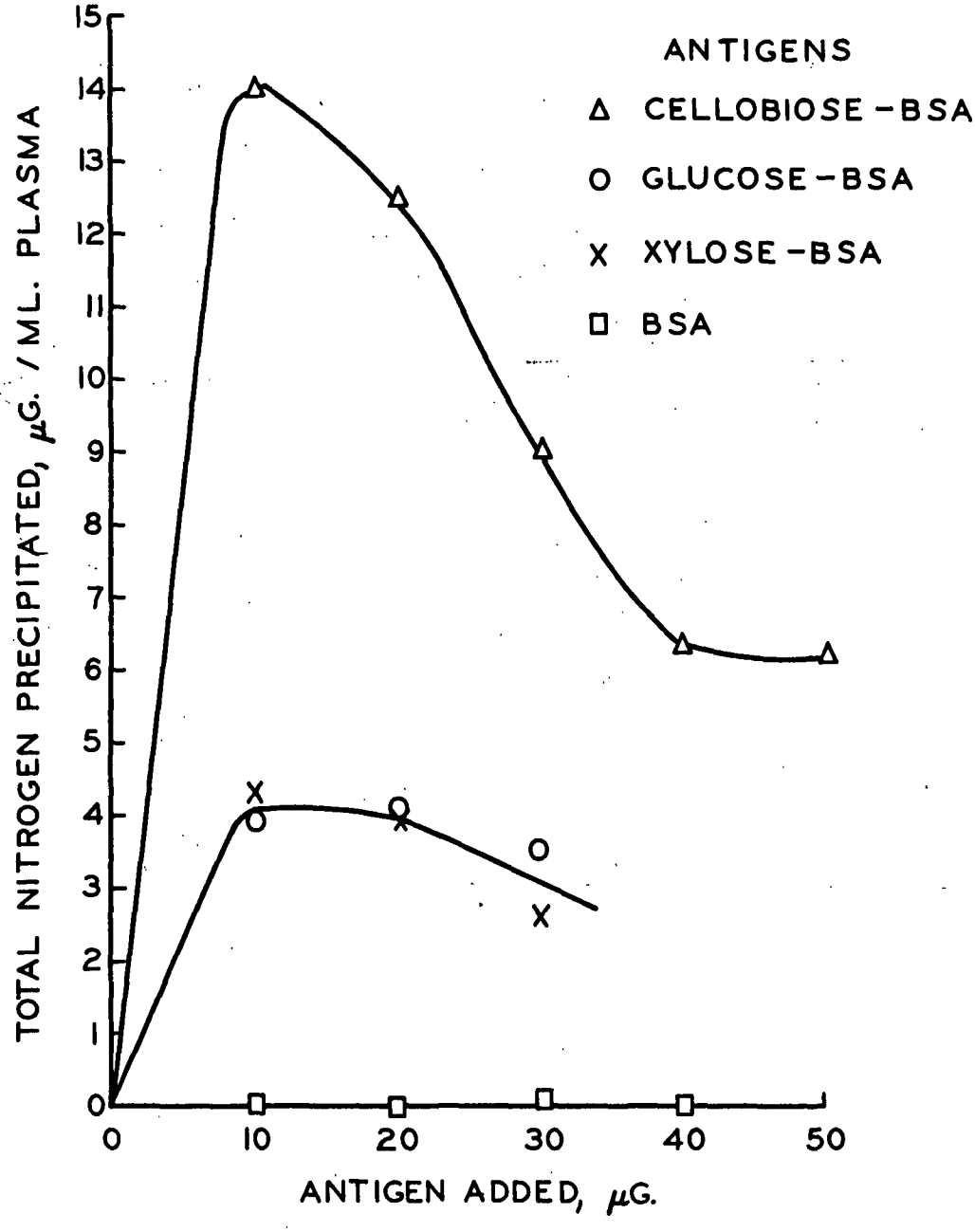


Figure 16. Quantitative Precipitin Tests: Absorbed Anti-cellobiose-BSA Plasma

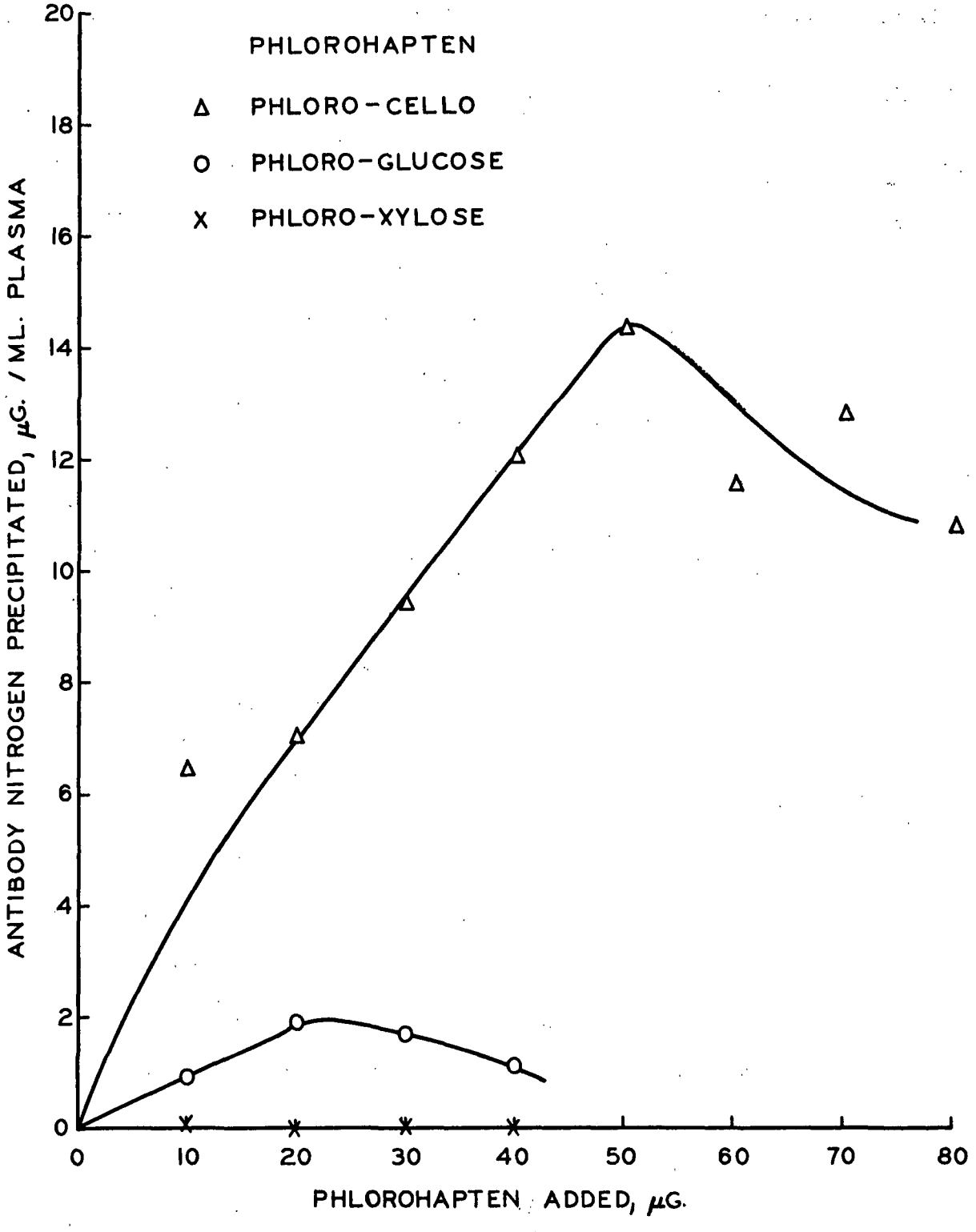


Figure 17. Quantitative Precipitin Tests: Anticellobiose-BSA Plasma

There was slight cross reactivity of the anticellobiose-BSA plasma with phloroglucose. There was no antibody response with phloroxylose despite the spontaneous precipitation of this hapten in the precipitin test, Table IV.

QUANTITATIVE HAPTEN INHIBITION STUDIES

Antixylose-BSA Plasma

For hapten inhibition studies 10 µg. of xylose-BSA were used as the precipitating antigen. The nitrogen content of 10 µg. of xylose-BSA was about 1 µg. N. Incomplete precipitation of this antigen with antibody in inhibition experiments could reduce the total nitrogen content of the precipitate by a maximum of less than 1 µg. N. The exact percent of antigen in the precipitate was not determined.

The error in the Folin-Ciocalteu determination of total nitrogen was ± 1 µg. N. Since in all inhibition studies the maximum response (no inhibition) was on the order of 10 µg. N, the values found for percent inhibition were only significant to ± 10%.

Table VI summarizes the results of hapten inhibition studies made with absorbed antixylose-BSA plasma.

All of the pentoses and the methyl pentose, L-rhamnose, produced inhibition at the 100 micromole level of addition. They were all weak inhibitors of precipitation. None of the hexoses or the disaccharides in Table VI produced significant inhibition at this high level of hapten addition. p-Aminophenyl-β-D-xylose, a hapten with essentially the same configuration as the antigenic determinant, was the most effective inhibitor.

There was not sufficient data for a detailed analysis of p-azophenyl-β-D-xyloside specificity of the antibodies. Inhibition by all the pentoses studied suggests a

lack of specificity in the combining site. But all of these haptens were weak inhibitors and the differences between percent inhibition values at the high level of hapten inhibition used were not highly significant. Yariv, et al. (28) and Allen and coworkers (29, 30) found that monosaccharides were weak inhibitors in quantitative hapten inhibition studies. Yariv, et al. (28) attributed this to the number of configurations the free monosaccharide can assume in solution. When only one particular configuration of the monosaccharide accommodates the antibody combining site, there is little inhibitory action on a molar basis.

TABLE VI

HAPTEN INHIBITION OF ABSORBED ANTIXYLOSE-BSA PLASMA[a]

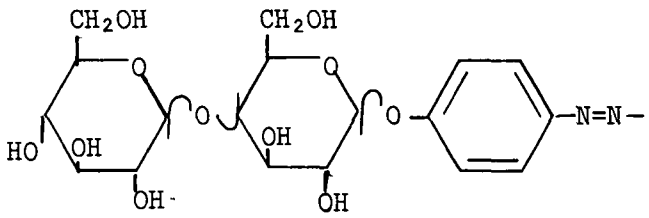
Hapten	Hapten Added, μ moles	Inhibition,[b] %
D-Xylose	100	60
D-Xylose	50	40
D-Xylose	10	29
L-Xylose	100	66
D-Ribose	100	15
L-Arabinose	100	20
L-Rhamnose	100	29
D-Glucose	100	0
D-Galactose	100	0
D-Mannose	100	0
Lactose	100	0
Sucrose	100	10
Maltose	100	0
Cellobiose	10	0
p-Aminophenyl-β-D-xylose	10	100

[a] Antixylose-BSA plasma used in 1.0-ml. amounts. Precipitating antigen — 10 μg. xylose-BSA.

[b] Error: ± 10%.

Anticellobiose-BSA Plasma

It was hypothesized that the antibody combining sites were complementary in configuration to a p-diazophenyl-β-D-cellobioside residue, illustrated below.



The validity of the hypothesis was tested with selected haptens, by determining the specificity of the antibodies for the terminal nonreducing glucose unit, the β linkage between glucose units, the point of attachment of the β linkage between the glucose units, the p-diazophenyl group, and the cellobioside molecule as a whole.

One set of inhibition studies was made with 1.0-ml. amounts of absorbed anticellobiose-BSA plasma using 10 μg. of cellobiose-BSA as the precipitating antigen. The remaining inhibition tests were all made with 1.0-ml. amounts of unabsorbed anticellobiose-BSA plasma and 50 μg. of phlorocello. Table VII gives a comparison of some of the results obtained for haptens studied in both systems. All haptens were added at 100 μmoles.

There was good agreement between the results in the two systems. The monosaccharides were all found to be poor inhibitors of precipitation. This was consistent with the findings of Yariv, et al. (28) and Allen and coworkers (29, 30).

The lack of inhibition with lactose (4-O-β-D-galactopyranosyl-D-glucopyranose) demonstrated specificity of the combining site for the terminal nonreducing glucose group. The terminal nonreducing galactose group of lactose cannot accommodate the

antibody site and does not produce inhibition. This was in agreement with the work of Goebel, et al. (23).

TABLE VII

HAPTEN INHIBITION OF ANTICELLOBIOSE-BSA PLASMA[a]

Hapten[b]	Absorbed Plasma Inhibition,[c] %	Unabsorbed Plasma Inhibition,[d] %
D-Xylose	0	5
L-Arabinose	9	16
D-Ribose	--	17
D-Glucose	9	10
D-Mannose	0	18
D-Galactose	9	--
Lactose	5	0
Sucrose	0	0
Maltose	36,52	40

[a]Anticellobiose-BSA plasma used in 1.0-ml. amounts.

[b]Hapten: 100 micromoles.

[c]Precipitating antigen — 10 μg. Cello-BSA; inhibition value error: ± 10%.

[d]Precipitating antigen — 50 μg. Phlorocello; inhibition value error: ± 10%.

Sucrose (α-D-glucopyranosyl-β-D-fructofuranose) contains a nonreducing glucose group but produced no inhibition. This demonstrated antibody specificity for the β anomeric linkage and/or the cellobioside molecule as a whole. The α-linked fructofuranose residue in sucrose does not fit into antibody combining sites directed toward a cellobioside group and does not therefore inhibit precipitation.

The most effective inhibitor in Table VII was maltose, 4-O-α-D-glucopyranosyl-D-glucopyranose. Maltose has the same configuration as cellobiose except for the α linkage between the glucose units. This structural difference makes maltose a weak inhibitor in the anticellobiose system and demonstrated the importance of the

β linkage between glucose units as a determinant of antibody specificity. The relative importance of maltose as an inhibitor is better illustrated in Fig. 18, a plot of percent inhibition values found for several haptens against the logarithm of the micromoles of hapten added in the inhibition test. Displacement of the curves along the logarithmic scale represent large changes in the amount of hapten required for inhibition.

The most effective inhibitor was p-nitrophenyl-β-D-cellobioside. The structure of this hapten was most similar to the configuration of the antigenic determinant group. Yariv, et al. (28) and Allen and coworkers (29, 30) also found that the p-nitrophenyl glycosides were the most potent inhibitors in antibody systems directed against p-diazophenyl-glycoside determinants.

Cellobiose was nearly as effective an inhibitor as the p-nitrophenyl derivative. This suggests that the p-diazophenyl group is relatively unimportant as a determinant of antibody specificity.

The importance of the β linkage between the glucose units of cellobiose was demonstrated by a comparison of inhibition with cellobiose and maltose and with β-methyl glucoside (β-methyl-D-glucopyranoside) and α-methyl glucoside (α-methyl-D-glucopyranoside). Cellobiose was approximately a thousandfold better inhibitor than maltose. Only 0.125 micromole of cellobiose was needed for 40% inhibition while 100 micromoles of maltose were needed for equivalent inhibition. β-Methyl glucoside was about 15 times as effective an inhibitor as α-methyl glucoside. For equivalent 30% inhibition three micromoles of β-methyl glucoside were required or 45 micromoles of α-methyl glucoside.

The importance of the cellobiose group as a determinant of specificity was demonstrated by a comparison of cellobiose and β-methyl glucoside inhibition. For

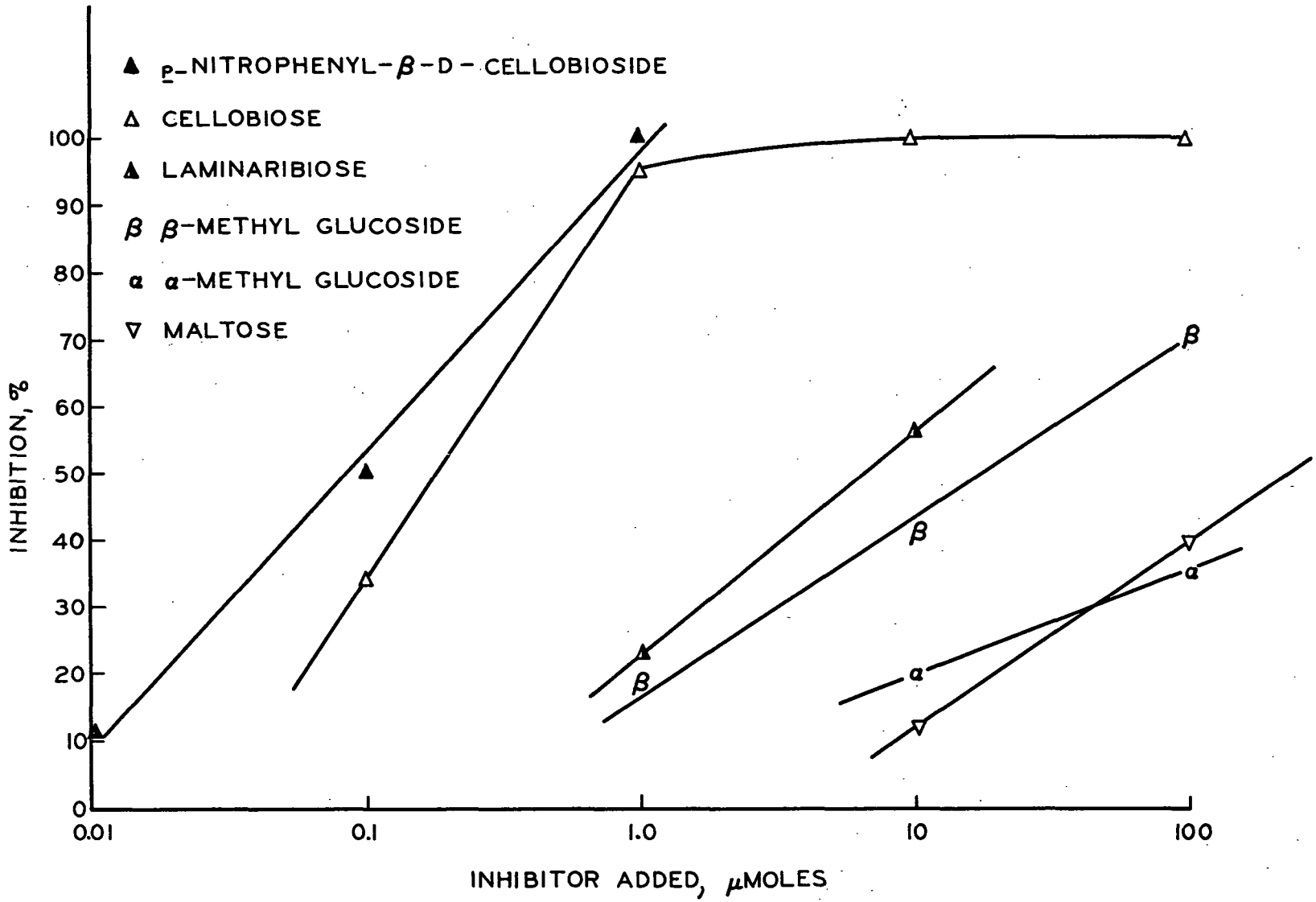


Figure 18. Quantitative Hapten Inhibition Studies. Anticellobiose-BSA Plasma

50% inhibition only 0.18 μmole of cellobiose was needed while 18 μmoles of β-methyl glucoside were required. Cellobiose was 100 times more potent an inhibitor. Antibodies were specific for both glucose units in the cellobiose structure. The presence of a β-linked methyl group on a nonreducing glucose unit did not accommodate the antibody combining site as well as a β-linked glucose group.

Maltose and α-methyl glucoside were nearly equivalent in their inhibitory ability. This indicated the antibody combining site could accommodate an α-glucosyl group about as well as an α-methyl group. Both were weak inhibitors.

Both α and β-methyl glucoside were better inhibitors than D-glucose. The presence of a methyl group at the anomeric carbon immunochemically was a significant alteration of structure.

Cellobiose, a β(1,4)-linked diglucose, was a more potent inhibitor of antibody precipitation than laminaribiose, β(1,3)-linked diglucose. The point of attachment of the β linkage between glucose units was therefore an important determinant of antibody specificity.

Laminaribiose was a slightly better inhibitor than β-methyl glucoside. The reducing glucose unit of laminaribiose, despite the β(1,3) linkage, more readily accommodated the antibody combining site than a β-linked methyl group. This suggests that specificity of the antibody for the cellobiose group as a whole was more important immunochemically than the position of the β linkage between the glucose units; at least for the moderate change of a β(1,3) linkage for a β(1,4).

An overall comparison of the inhibition data indicates the relative importance of each portion of the antigenic determinant toward which antibody specificity was directed. In decreasing order of importance these were: the terminal nonreducing

glucose group, the β linkage between glucose units, the cellobiose group as a whole, the position of the β linkage between the glucose units, and the p-diazophenyl group.

There was a close correspondence between the results obtained in inhibition studies with chicken anticellobiose serum and the results obtained by Gleich and Allen (29) for the same precipitating system using rabbit serum. Despite the low antibody response found with chickens, the specificity characteristics of the antibodies were nearly the same as those found for rabbit antibodies.

CONCLUSIONS

All experiments were made with hyperimmune chicken gamma globulins or with sorbed or whole plasma in physiological saline. This was the first study of the chicken immune system where the complications of gamma globulin aggregation in high salinity and macroglobulin antibodies were recognized and avoided.

Although the antibody response in chickens to p-azophenyl-β-D-glucoside, -xyloside, and -cellobioside determinant groups was weak, it was specific. The presence of the hydroxymethyl group at carbon five in glucose compared with xylose was a significant alteration in structure immunochemically to confer antibody specificity.

The amount of antibody in chickens specific for a particular glycosidic group was much lower than that found in rabbits to the same glycosidic group. In terms of quantity, chickens were inferior producers of precipitating antihapten antibodies.

The relative importance in decreasing order of each portion of the p-diazophenyl-β-D-cellobioside determinant in conferring antibody specificity was:

the terminal nonreducing glucose group
the β linkage between glucose units
the cellobiose group
the position of the β linkage between glucose units
the p-diazophenyl group

It was concluded that the specificity characteristics of the chicken antibodies were similar to those of rabbits for this same immunochemical system.

SUGGESTIONS FOR FUTURE WORK

In the chicken immune system the most interesting phenomenon is the aggregation of gamma globulins in 8% NaCl. Additional studies should be made to determine whether aggregation occurs with salts other than NaCl and at NaCl concentrations intermediate between 0.9 and 8%. Eventually, it would be of interest to determine the nature of the chemical and physical forces involved in the aggregation.

Additional studies of antihapten antibodies in chickens are undesirable due to the low response of precipitating antibody. Further work should be done with the xylose determinant, perhaps in the rabbit. The demonstration of specificity for the xylose determinant in the chicken immune system suggests that specific antibody would also be produced in rabbits. An antibody population specific for a terminal nonreducing xylose residue could be absorbed with glucose-BSA and labelled with a fluorescein dye. The immunofluorescent staining of wood fibers and tissue sections with the labelled antibody should locate terminal xylose residues and would be evidence for the location of xylan hemicelluloses. Similar labelling and staining with cellobiose specific antibody and control globulins would also be needed for comparison.

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APPENDIX I

SYNTHESIS OF p-AMINOPHENYL-β-D-CELLOBIOSIDE

The reaction sequence used for the synthesis of p-aminophenyl-β-D-cellobioside is illustrated in Fig. 19.

α-Cellobiose octaacetate was obtained from Eastman Organic Chemicals. A procedure described by Schroeder and Morak (40) was used to form acetobromo cellobiose. To 14.0 g. of cellobiose octaacetate were added 20 ml. of 30-32% HBr in glacial acetic acid and 20 ml. of dichloroethane. The octaacetate was worked up in the acid mixture by stirring with a glass rod. When most of the lumps of octaacetate were broken up, the mixture was placed on a mechanical shaker. After two hours the reaction mixture was dissolved in 64 ml. of chloroform and transferred to a separatory funnel. The chloroform solution was washed three times with cold distilled water, once with dilute sodium bicarbonate solution, and twice again with cold distilled water. The chloroform layer was poured into a flask containing anhydrous calcium chloride and dried for one-half hour. The solution was filtered, warmed slightly, and petroleum ether added to incipient turbidity. This solution was stored in the cold for 4 hours. The crystals that formed were filtered and dried in a vacuum desiccator. The mother liquor was concentrated on a rotary evaporator, warmed slightly, and petroleum ether again added to turbidity. The suspension was stored overnight at 4°C. The crystals formed were filtered and dried in a vacuum desiccator. Yield was 4.04 g. from the first crop, 8.36 g. from the second crop of crystals. Total yield 12.40 g.; 86% of theoretical; m.p. 178-9°C.; lit. (41), ca. 180°C.

The p-nitrophenylation procedure of Babers and Goebel (42) was found to give the highest yields for this reaction step. p-Nitrophenol was obtained from Eastman Organic Chemicals and recrystallized from hot water. In a typical reaction 2.95 g.

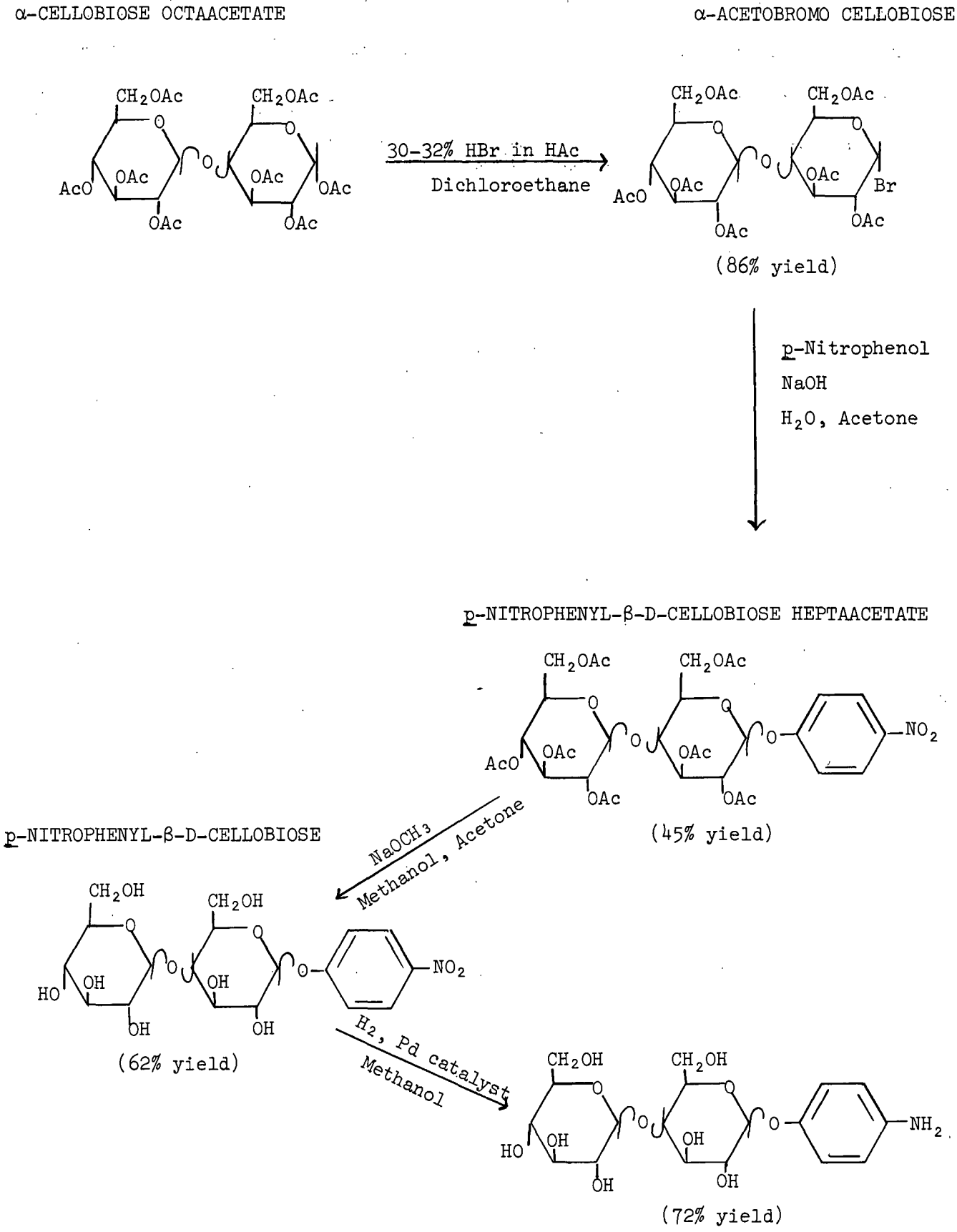

Figure 19. Synthesis of p-Aminophenyl-β-D-cellobioside

of p-nitrophenol and 0.85 g. of NaOH were dissolved in a mixed solvent of 25 ml. of acetone and 20 ml. of distilled water. To this was added 7.40 g. of acetobromo cellobiose dissolved in 50 ml. of acetone. Molar ratios of p-nitrophenol:NaOH:acetobromo cellobiose were 2:2:1. The solution was placed on a mechanical shaker for 10 hours.

After this reaction time the acetone was removed on a rotary evaporator leaving a bright yellow precipitate. About 50 ml. of distilled water were added and the precipitate was filtered and washed with several 100-ml. portions of distilled water to remove the yellow color. The residual precipitate was suspended in hot methanol, then stored overnight at 4°C. The precipitate formed was filtered and dried in a vacuum desiccator. Concentration of the methanol filtrate on a rotary evaporator produced additional precipitation of product. This was also filtered and dried. Yield 3.74 g.; 45% of theoretical; m.p. 233-5°C.; (lit. 42), 234-5°C.

For deacetylation of the p-nitrophenyl-β-D-cellobiose heptaacetate a modification of the procedure of Thompson and Wolfrom was used (43). About 1.26 g. of the heptaacetate were dissolved in a mixed solvent of 20 ml. of acetone and 20 ml. of methanol. To this was added 1.30 ml. of 0.1N sodium methoxide solution. After stirring for one hour at room temperature, 20 ml. of distilled water and about 5 grams of Rexyn IRG 501 ion exchange resin were added. This suspension was slurried for 10 minutes, then filtered to remove the resin. The filtrate was concentrated on a rotary evaporator. Absolute ethanol was added to the filtrate to aid in water removal on the evaporator. The final sirup was dissolved in 5 ml. of hot methyl cellosolve and stored overnight in the cold room. The platelike crystals formed were filtered and dried in a vacuum desiccator. Yield 0.48 g.; 62% of theoretical; m.p. 244-6°C.; lit. (29), 245-6°C.

The final step in the synthesis was the reduction of the p-nitrophenyl compound to the p-aminophenyl derivative. For this 0.77 g. of the p-nitrophenyl-β-D-cellobioside were placed in a thick-walled glass bottle with 0.42 g. of 10% palladium on activated charcoal catalyst and 30 ml. of methanol. This suspension was placed on a hydrogenation apparatus, the glass reaction vessel evacuated, then pressurized with hydrogen gas at about 20 p.s.i.g. After agitating for one hour at room temperature the hydrogen gas was released. The suspension was filtered to recover the catalyst and the methanol filtrate evaporated to dryness on a rotary evaporator. The solids were dissolved in 10 ml. of hot ethanol and stored at 4°C. overnight. The precipitate formed was filtered and dried in a vacuum desiccator. Yield 0.52 g.; 72% of theoretical; m.p. partial collapse at 150°C., final melting at 241°C.; lit. (29), partial collapse 143°C., final melting 238-9°C.

APPENDIX II

QUANTITATIVE PRECIPITIN AND HAPTEN INHIBITION STUDIES

Frozen plasma pools were thawed overnight in the cold room and always contained a flocculated material that was removed by centrifugation at about 4000 x g. The plasma was further clarified by centrifugation in a preparative ultracentrifuge (Beckman Instruments, Model L-2, Type 30 Rotor) at an average force of about 35,000 x g. for 4 hours at 4°C. Lipids present in the plasma were concentrated at the surface and were removed by aspiration. The plasma was decanted from small amounts of gelatinous precipitates and recentrifuged at about 35,000 x g for 2 hours at 4°C. The surface layers were skimmed by aspiration and the decanted plasma used in precipitin or inhibition studies.

Except for Table V, p. 47, a constant volume of plasma was used with varying amounts of antigen in precipitin tests. The plasma volume used depended upon the magnitude of the response expected. Unabsorbed plasma had about 100-150 µg. antibody nitrogen per ml. precipitatable by BSA antigens. Since the Folin-Ciocalteu colorimetric test was only valid in the range of 0-35 µg. nitrogen, plasma volumes of 0.25 ml. were used in precipitin tests of this type. Absorbed plasma gave a much lower response; 5-15 µg. N. This was also true for tests made with the phloroglucinol haptens. In these tests 1.0-ml. volumes of plasma were used.

Antigen solutions were prepared at a concentration of 100 µg./ml. by dissolving the lyophilized antigen in a 0.05M phosphate buffered physiological saline solution, pH 7.2. For the precipitin test, 13 x 100 mm. test tubes were used. Antigen solution was added to duplicate tubes using a 1-cc. tuberculin syringe. In the routine test each tube in the series received an increasing volume increment of 0.10 ml., yielding final antigen concentrations of 10, 20, 30, etc. µg. The total volume in each tube was adjusted to 1.0 ml. by addition of 0.05M phosphate buffered physiological

saline solution. Another set of duplicate tubes containing only physiological saline were the controls for the test. A uniform volume of antiserum was then added to all tubes. The tubes were capped with parafilm and thoroughly mixed on a Vortex Jr. Mixer. After one hour at room temperature the tubes were again mixed, then stored at 2-4°C.

In some tests with BSA antigens and unabsorbed plasma the tubes were stored for only 48 hours. In most tests, however, the tubes were kept at 4°C. for 96 hours (4 days). Tubes were mixed twice daily during storage. The tubes were then centrifuged at about 4,000 x g at 4°C. for 30 minutes. The supernatants were decanted and the precipitates washed by adding 1-2 ml. of cold 0.9% saline solution per tube. Each tube was agitated on the Vortex Mixer to break up the precipitate and insure thorough washing. The tubes were centrifuged at 4,000 x g for 20 minutes and the washings repeated. After centrifuging at 4,000 x g for 20 minutes more, the supernatant washings were decanted and discarded. The tubes containing the precipitates were inverted and drained overnight in the cold room.

A similar procedure was used for hapten inhibition tests. Hapten solutions were prepared in 0.05M phosphate buffered physiological saline solution. The concentration of hapten used depended upon the amount of hapten that was available or on the extent of inhibition expected. Stock solutions of commercially available haptens were prepared at 100 micromoles/ml. concentration.

For inhibition experiments, hapten (inhibitor) solutions were placed in duplicate 13 x 100-mm. test tubes. The solution volume was adjusted to 1.0 ml. with 0.05M phosphate buffered physiological saline solution. Plasma was then added in 1.0-ml. amounts per tube. The tubes were capped with parafilm, agitated on the Vortex Mixer, and allowed to react for one-half hour at room temperature. The parafilm was removed and the precipitating antigen, 50 µg. of phlorocello in 0.5 ml.

was added to each tube. The tubes were again capped with parafilm, mixed, and allowed to react for one hour at room temperature. Tubes were again mixed and stored at 2-4°C. for 96 hours. In each inhibition study two sets of duplicate controls were used; one as above without inhibitor and one as above without inhibitor or antigen.

Centrifugation and washing procedures for hapten inhibition studies were the same as those used in quantitative precipitin tests.

APPENDIX III

FOLIN-CIOCALTEU DETERMINATION OF PROTEIN NITROGEN

The procedure used was a modification of the methods of Lowry, et al. (44) and Mage and Dray (45). The following reagents were employed:

0.05N NaOH

1% $CuSO_4 \cdot 5H_2O$

2% Sodium citrate

2% Na_2CO_3 in 0.10N NaOH

0.9% Saline solution

Phenol reagent, 2N (Fisher Scientific Co.)

Washed precipitates in test tubes were each dissolved in 0.30 ml. of 0.05N NaOH solution. Fresh copper sulfate reagent was prepared by mixing equal parts of the 1% copper sulfate solution and the 2% sodium citrate solution. This reagent was added to the 2% Na_2CO_3 solution in the proportions of 1 ml. to 50 ml. of the carbonate solution.

When the precipitates were completely dissolved, 1.5 ml. of the alkaline copper solution and 3.0 ml. of 0.9% saline solution were added to each test tube. The tubes were stoppered, agitated on a Vortex Mixer, and allowed to react for one-half hour.

Phenol reagent was diluted 1:1 with distilled water and added in 0.15-ml. amounts per tube. Immediately upon phenol reagent addition the tubes were capped and thoroughly agitated on the Vortex Mixer. After one-half hour the optical densities (O.D.'s) of the solutions were read at 750 nm. (Beckman Instruments, Inc., DU Spectrophotometer). The optical densities were compared with the O.D. of a blank that received the same sequential treatment with all the reagents.

Normally, a 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium tartrate solution is used in the Folin-Ciocalteu test. This reagent was difficult to prepare and unstable. Eggstein and Kreutz (46) found improved reagent stability when sodium citrate replaced the sodium tartrate. This reagent was found to degrade after 3 or 4 weeks. Separate copper sulfate and sodium citrate solutions were stable for several months.

Color development with phenol reagent varies with different proteins and is not strictly proportional to the concentration of protein (44). The phenol reagent must be calibrated for the particular protein being investigated; in this case chicken gamma globulins. The globulin fraction obtained by the ammonium sulfate precipitation procedure of Campbell, et al. (32) was used as the reference material. A standard solution of this protein was prepared and the nitrogen content determined by the Kjeldahl procedure (47). This standard solution was then used to prepare the calibration curve for the Folin-Ciocalteu test.

Samples of the protein standard solution were tested with each Folin nitrogen determination. When the nitrogen value for the reference solution declined, the copper sulfate and citrate solutions were discarded or fresh phenol reagent was purchased. Allen (48) found that the maximum shelf life of the phenol reagent was about 6 months. Each time fresh phenol reagent was purchased a new calibration curve was made.

Figure 20 shows the three calibration curves used in the course of this work. As expected, optical density was not directly proportional to concentration for the chicken gamma globulin. There were large variations in the calibration curves for the different commercial samples of phenol reagent. The magnitude of the variations was greater at higher nitrogen concentrations.

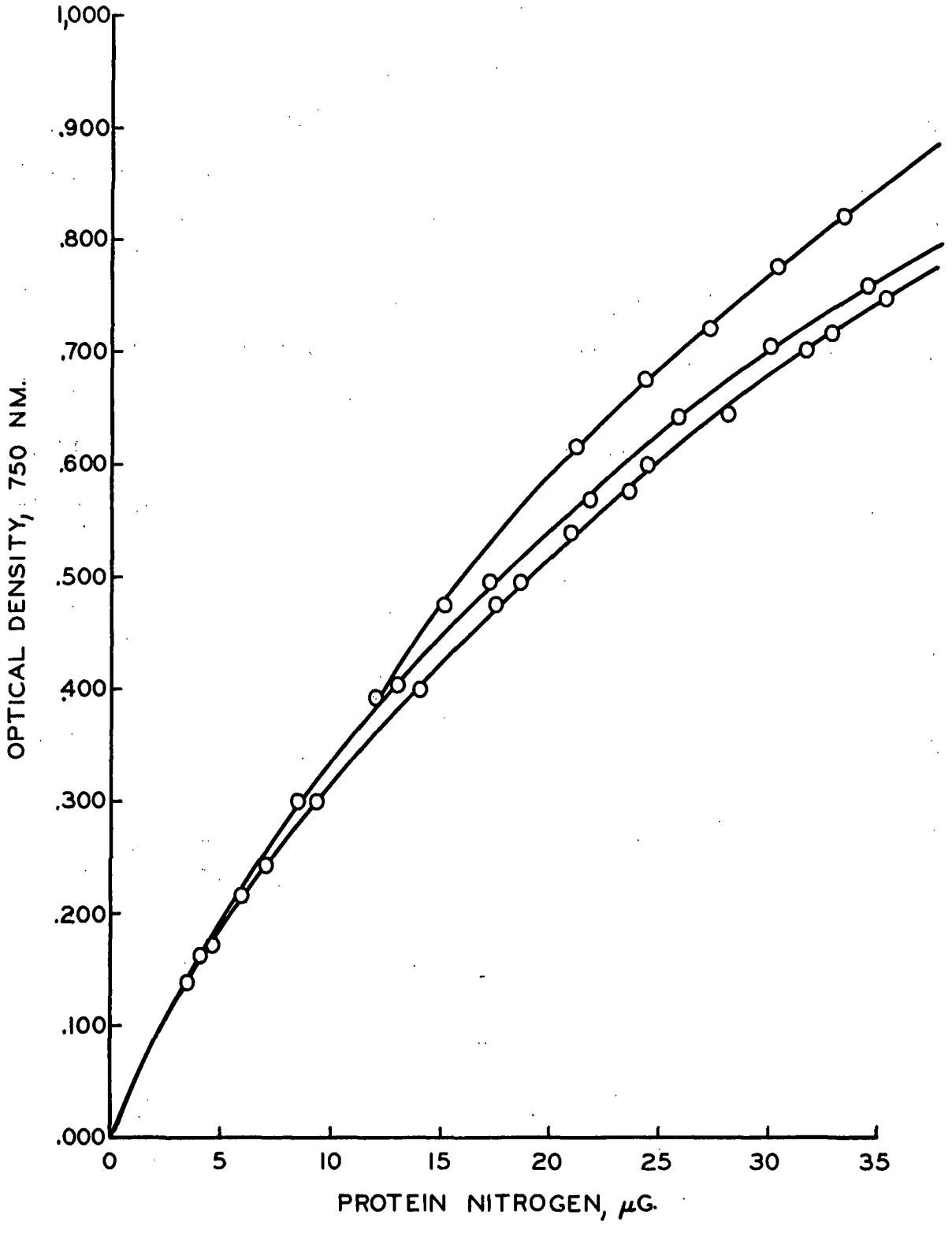

Figure 20. Calibration Curves for Phenol Reagent in Folin-Ciocalteu Test

APPENDIX IV

DETERMINATION OF PHLOROGLUCINOL HAPTEN NITROGEN

The procedure used was similar to that described by Yariv, et al. (28) and Gleich and Allen (29). For these determinations a stock buffer solution of 0.20M tris(hydroxymethyl)aminomethane (TRIS or Tham, Fisher Scientific Co.) was prepared. The pH of the TRIS solution was adjusted to 8.0-8.1 with concentrated HCl.

Washed precipitates in test tubes were dissolved in 0.10 ml. of 0.10N NaOH solution. The volume in each tube was adjusted to 2.5 ml. with TRIS-HCl buffer containing a 0.05M concentration of the sugar corresponding to the specificity. That is, if anticellobiose-BSA sera were used in the precipitin or hapten inhibition test, the TRIS-HCl buffer would be 0.05M in cellobiose. The optical densities of the solutions were read at 280 and 400 nm. From these data, antigen, antibody, and total nitrogen precipitated were estimated from calibration curves for the three phloroglucinol haptens.

Figure 21 is the calibration curve for optical density at 280 nm. vs. micrograms of protein nitrogen. The chicken gamma globulin fraction used as a standard in the Folin-Ciocalteu nitrogen determination was used for this calibration. The curve was linear in the range of protein concentration studied, slope:

$$\text{O.D. at 280 nm./}\mu\text{g. protein N} = 141$$

There was no absorption by the protein standard at 400 nm.

Figures 22, 23, and 24 are the calibration curves, O.D. at 400 and 280 nm. vs. concentration, µg., for phlorocello, phloroglucose, and phloroxylose, respectively. In each case the phlorohaptens were prepared in TRIS-HCl buffer containing the corresponding carbohydrate. The curves are calibrated for optical density at

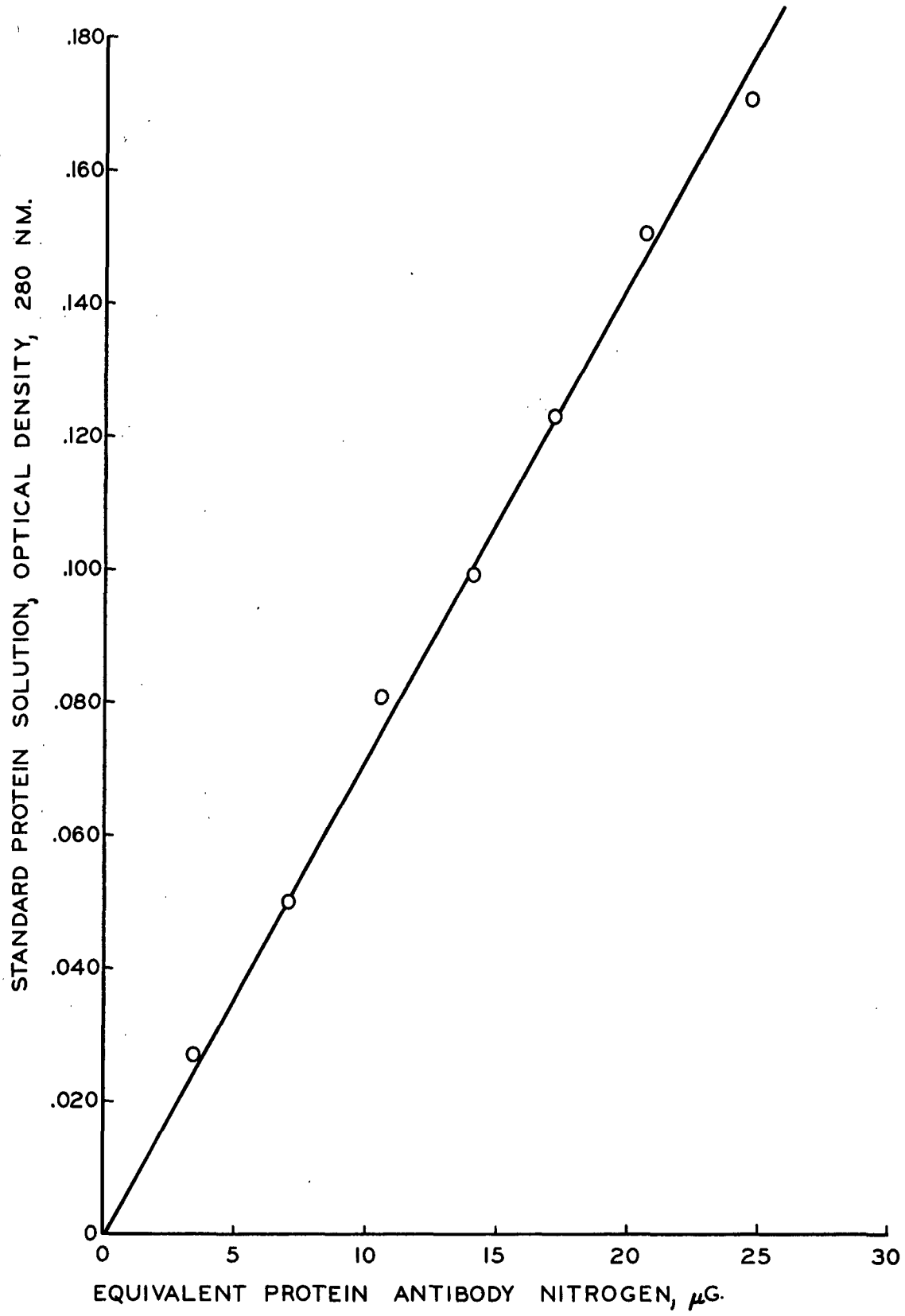

Figure 21. Calibration Curve

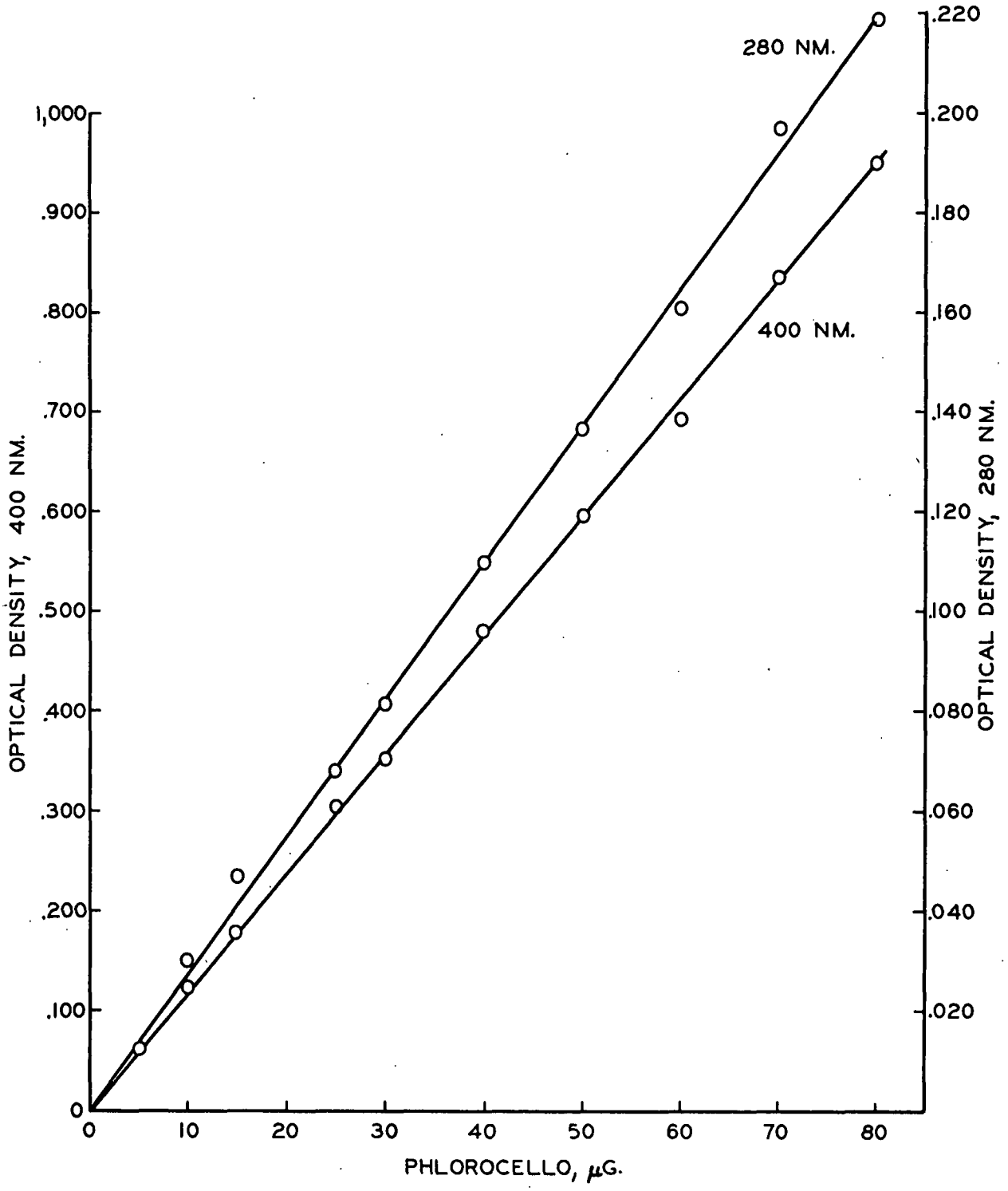

Figure 22. Calibration Curve for Phlorocello

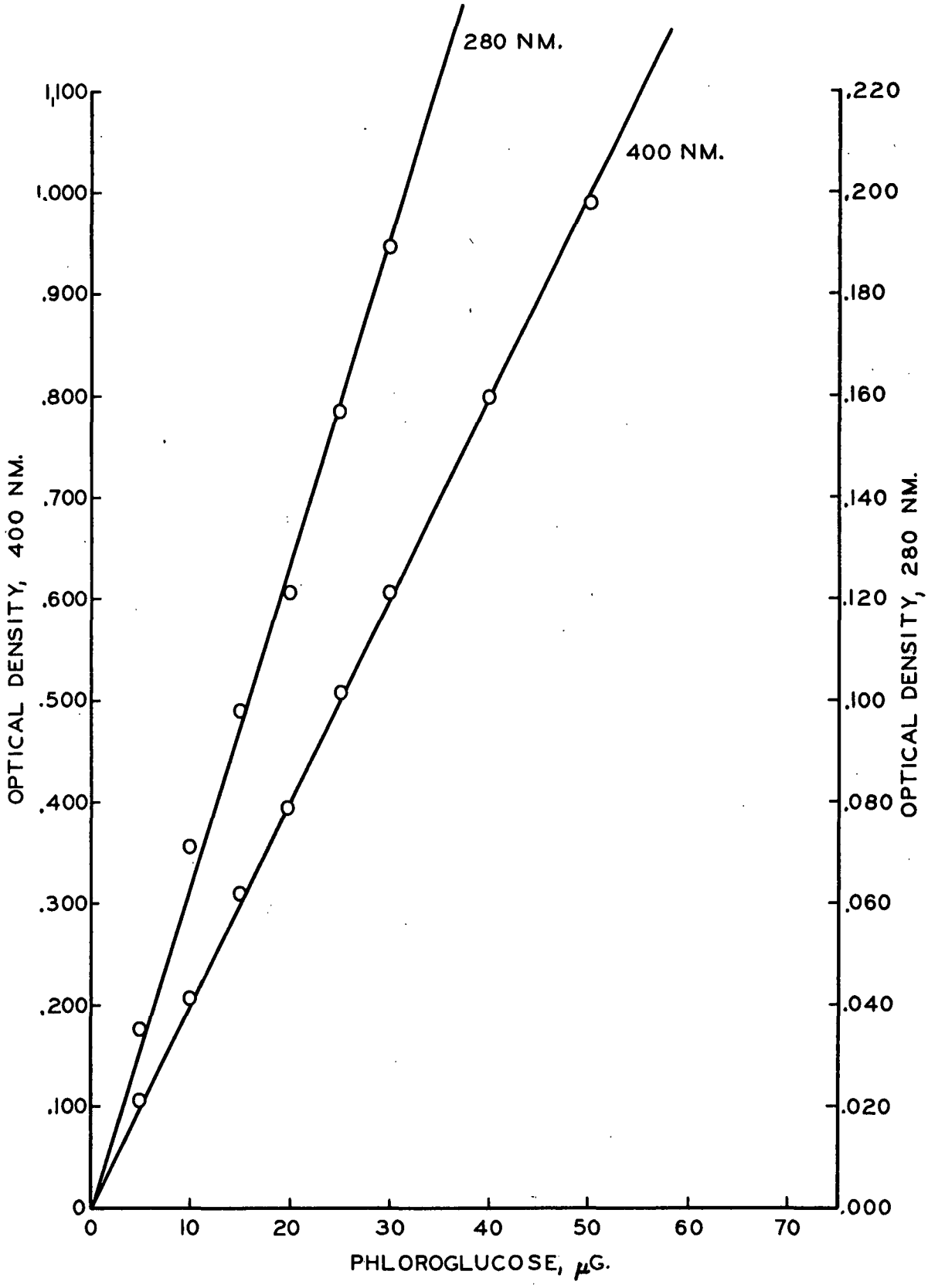

Figure 23. Calibration Curve for Phloroglucose

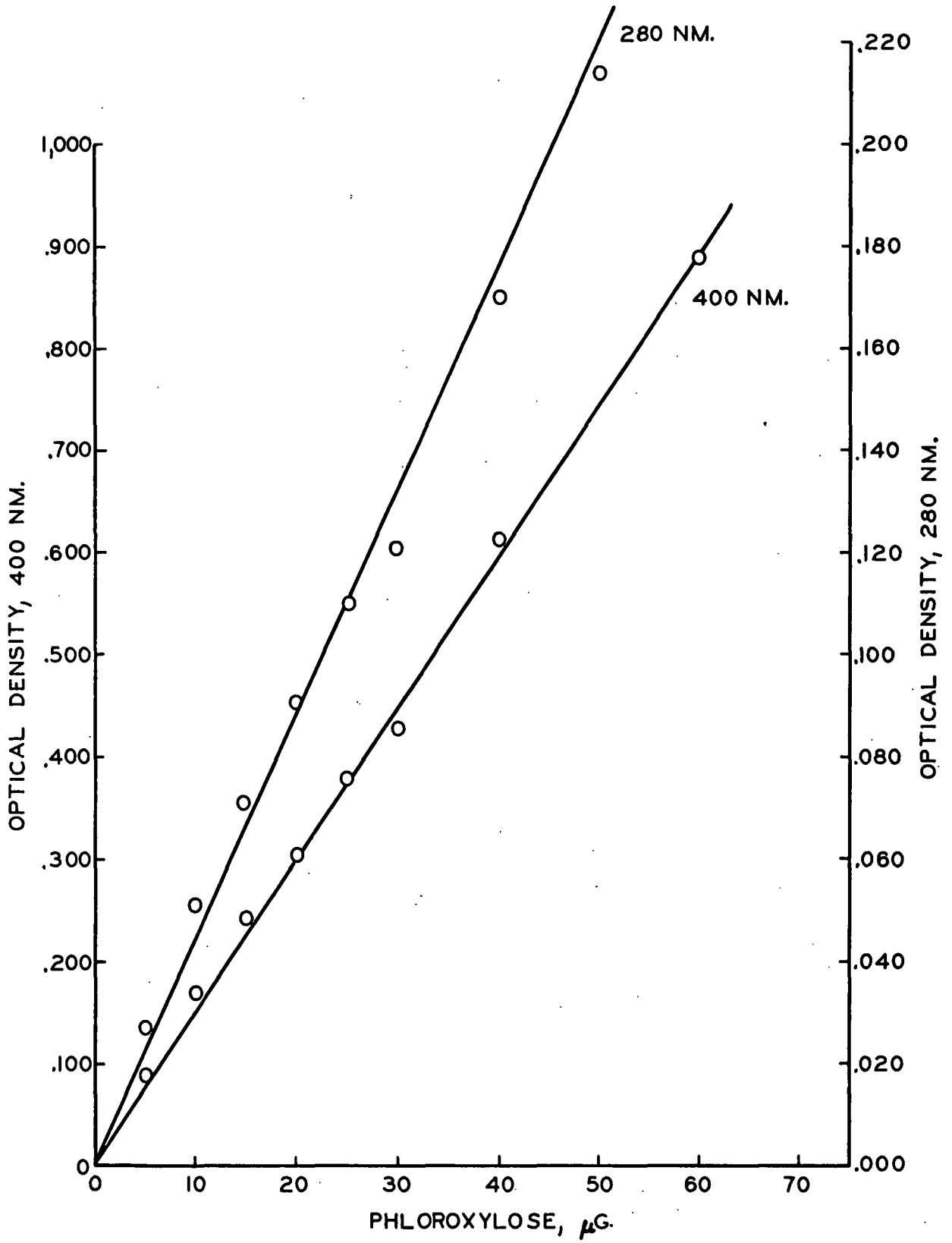

Figure 24. Calibration Curve for Phloroxylose

400 nm. against the amount of phlorohapten in micrograms. The optical density readings at 280 nm. for these haptens are assumed to be a measure of micrograms of hapten or antigen nitrogen. All the curves are linear in the range studied.

Tables VIII and IX using standard solutions demonstrate how this procedure was used to determine antigen, antibody, and total nitrogen.

Three different volumes of chicken gamma globulin standard solution were used with four different amounts of phlorocello added at each volume level. The phlorocello solution was prepared in TRIS-HCl-cellobiose buffer. Each tube received 0.10 ml. of 0.10N NaOH solution and was adjusted to 2.5 ml. with TRIS-HCl-cellobiose buffer. Optical densities were read at 280 and 400 nm. From the phlorocello calibration curve the equivalent O.D. at 280 nm. was determined at the observed O.D. at 400 nm. Subtracting this estimated O.D., 280 nm., attributable to phlorocello from the total O.D. at 280 nm., gave the net O.D., 280 nm., attributable to antibody protein. This value was converted to micrograms of nitrogen from the calibration curve given in Fig. 21.

The following relationships apply:

O.D., 280 nm. :: total µg. nitrogen

O.D., 400 nm. :: µg. phlorohapten or antigen

O.D., 400 nm. x 0.435 = O.D., 280 nm. attributable to phlorocello

= 280 nm. correction factor

observed O.D., 280 nm. - 280 nm. correction factor = net O.D., 280 nm., attributable to antibody protein

net O.D., 280 nm. :: µg. antibody nitrogen, Fig. 21.

TABLE VIII

QUANTITATIVE DETERMINATION OF ANTIBODY NITROGEN

Standard Solutions						
Globulin Nitrogen Added, µg.	Phlorocello, µg.	O.D., 280 nm.	O.D., 400 nm.	Correction Factor, 280 nm.	Corrected O.D., 280 nm.	Antibody N, µg.
3.5	10	0.055	0.122	0.028	0.027	3.8
3.5	20	0.077	0.236	0.050	0.027	3.8
3.5	30	0.108	0.354	0.081	0.027	3.8
3.5	40	0.129	0.447	0.103	0.026	3.7
10.5	10	0.100	0.120	0.027	0.073	10.3
10.5	20	0.131	0.242	0.055	0.076	10.7
10.5	30	0.156	0.353	0.081	0.075	10.6
10.5	40	0.174	0.447	0.102	0.072	10.2
17.5	10	0.151	0.122	0.028	0.123	17.3
17.5	20	0.174	0.240	0.055	0.119	16.8
17.5	30	0.206	0.355	0.081	0.125	17.6
17.5	40	0.226	0.452	0.103	0.123	17.3

TABLE IX

QUANTITATIVE DETERMINATION OF PHLOROCELLO

Standard Solutions				
Globulin Nitrogen Added, µg.	Phlorocello, µg.	Total N, µg.	Phlorocello Amount, µg.	Phlorocello Nitrogen, µg.
3.5	10	7.8	10.3	3.9
3.5	20	10.9	19.8	7.0
3.5	30	15.2	29.7	11.4
3.5	40	18.2	37.6	14.5
10.5	10	14.1	10.1	3.8
10.5	20	18.5	20.3	7.8
10.5	30	22.0	29.6	11.4
10.5	40	24.5	37.6	14.4
17.5	10	21.3	10.3	3.9
17.5	20	24.5	20.1	7.8
17.5	30	29.0	29.8	11.4
17.5	40	31.8	37.9	14.5

There was good agreement between the known µg. of nitrogen in the reference solutions and the values predicted from the calibration curve. Except for the highest addition (40 µg.), there was good agreement between known amounts of phlorocello added and the values determined from the optical density readings at 400 nm. With the 40-µg. additions the predicted values were low by about 2 µg. The ratios of O.D., 400 nm./O.D., 280 nm. for the three phlorohaptens were obtained from the calibration curves. These ratios were 0.435, 0.320, and 0.298 for phlorocello, phloroglucose, and phloroxylose, respectively.